

From DEPARTMENT OF MEDICINE, SOLNA
Karolinska Institutet, Stockholm, Sweden

LINEAGE STABILITY AND EFFECTOR FUNCTIONS OF CD4+FOXP3+ REGULATORY T CELLS

Christina Seitz



**Karolinska
Institutet**

Stockholm 2019

All previously published papers were reproduced with permission from the publisher.

Published by Karolinska Institutet.

Printed by Eprint AB 2019

© Christina Seitz, 2019

ISBN 978-91-7831-458-4

LINEAGE STABILITY AND EFFECTOR FUNCTION OF CD4+FOXP3+ REGULATORY T CELLS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Christina Seitz

Principal Supervisor:

Associate Professor John Andersson
Karolinska Institutet
Department of Medical Epidemiology and
Biostatistics

Opponent:

Associate Professor Gislaine Martins
Cedars-Sinai Medical Center
Department of Medicine

Co-supervisors:

Associate Professor Andreas Lundqvist
Karolinska Institutet
Department of Oncology-Pathology
Cancer Center Karolinska

Examination Board:

Associate Professor Antonio Gigliotti Rothfuchs
Karolinska Institutet
Department of Microbiology, Tumor and Cell
Biology

Associate Professor Lisa Westerberg
Karolinska Institutet
Department of Microbiology, Tumor and Cell
Biology

Professor Maria Faresjö
Jönköping University
Department of Natural Science and Biomedicine
School of Health and Welfare

Professor Richard Kennedy
Queen's University of Belfast
Centre for Cancer and Cell Biology
School of Medicine, Dentistry and Biomedical
Sciences

Professor Anna-Lena Spetz
Stockholm University
Department of Molecular Biosciences
The Wenner-Gren Institute

ABSTRACT

This work focuses on regulatory T (Treg) cell biology in the context of defining the role of forkhead box protein 3 (FOXP3) isoform expression and Treg mediated suppression via CTLA-4 (cytotoxic T-lymphocyte-associated protein 4).

Treg cells are crucial in maintaining immunological tolerance and they in part act by suppressing dendritic cells (DCs). Previous studies have demonstrated that Treg cells express the co-inhibitory receptor CTLA-4, which is essential for Treg cells ability to control the expression of the costimulatory molecules CD80/CD86 on DCs. In **study I** we generated a novel mouse strain that exclusively expresses the naturally occurring ligand-independent CTLA-4 isoform (liCTLA-4) in Treg cells only. This isoform cannot control CD80/CD86 expression by direct binding to these molecules. One of the key findings in this study is that these *Ctla4^{ex2fl/fl}Foxp3-Cre* mice did not develop a lymphoproliferative phenotype early in life. When we extended our study, we saw that older *Ctla4^{ex2fl/fl}Foxp3-Cre* mice (6 months) developed an inflammatory phenotype in the lung. Interestingly, *Ctla4^{ex2fl/fl}Foxp3-Cre* mice had an increased number of Treg cells, in particular Treg cells lacking CD25 expression. The dramatic increase of Treg cells could potentially compensate for any functional impairment due to altered CTLA-4 expression. Thus, we isolated Treg cells for *in vitro* assays to further investigate Treg cell function using an DC suppression assay that we established. We found that Treg cells isolated from *Ctla4^{ex2fl/fl}Foxp3-Cre* were able to control CD80 and CD86 expression. However, Treg cells isolated from *Ctla4^{ex2fl/fl}Foxp3-Cre* failed to support upregulation of PD-L2 on suppressed DCs.

The transcription factor FOXP3 is a key regulator for Treg cell differentiation and is required to maintain their suppressive phenotype. FOXP3 occurs as four distinct isoforms, full-length FOXP3 and isoforms lacking exon 2 and or exon 7. In **study II** we determined the FOXP3 isoform expression in plaques and blood from patients suffering from atherosclerotic disease. We found a positive correlation between FOXP3 Δ 2 splice variant expression and plaque stability in plaque tissue that was not apparent in blood. Another key finding from this study is that during Treg cell activation the overall upregulation of FOXP3 expression is mainly due to an increased expression of the isoform FOXP3 Δ 2. In **study III and IV** we reported on two patients with a novel frameshift mutation in the FOXP3 gene (NM_014009.3:c.305delT) located in exon 2 which results in a premature stop codon and consequently the loss of any isoforms expressing exon 2. Both patients had a very mild IPEX phenotype, which strongly suggests FOXP3 Δ 2 alone is able to at least partially maintain Treg cell development and function. Investigating the female carrier, we saw approximately 20% of the Treg pool had the c.305delT mutation not matching the expected 50% from random X-chromosome inactivation. RNA sequencing allowed us to identify a set of genes, several which previously have been demonstrated to regulate FOPX3 expression, that are specifically regulated by full-length FOXP3. This gene set may explain the loss of c.305delT Treg cells and we currently favor the view that they regulate the lineage stability of Treg cells.

LIST OF SCIENTIFIC PAPERS

- I. **Christina Seitz***, Sang Liu*, Katrin Klocke, Anne-Laure Joly, Paulo V Czarnewski, Christopher A Tibbitt, Sara M Parigi, Lisa S Westerberg, Jonathan M Coquet, Eduardo J Villablanca, Kajsa Wing, John Andersson. Multi-faceted inhibition of dendritic cell function by CD4+Foxp3+ regulatory T cells. Journal of Autoimmunity 98:86-94, 2019.
- II. Anne-Laure Joly, **Christina Seitz***, Sang Liu*, Nikolai V Kuznetsov, Karl Gertow, Lisa S Westerberg, Gabrielle Paulsson-Berne, Göran K Hansson, John Andersson. Alternative splicing of FOXP3 controls regulatory T cell effector functions and is associated with human atherosclerotic plaque stability. Circulation Research 122:1385–1394, 2018.
- III. Katie Frith, Anne-Laure Joly, Cindy S Ma, Stuart G Tangye, **Christina Seitz**, Zuzana Lohse, Charles F Verge, John Andersson & Paul Gray. The FOXP3Δ2 isoform supports regulatory T cell development and protects against severe IPEX. The Journal of Allergy and Clinical Immunology 144:317-320, 2019.
- IV. **Christina Seitz**, Anne-Laure Joly, Katie Frith, Zuzanna Lohse, Paul Gray, John Andersson. FOXP3fl controls the lineage-stability of CD4+FOXP3+ regulatory T cells. Manuscript

* denotes shared authorship.

TABLE OF CONTENTS

<u>1</u>	<u>INTRODUCTION</u>	<u>1</u>
1.1	THE IMMUNE SYSTEM	1
1.2	T CELLS	2
1.2.1	THYMIC DEVELOPMENT	3
1.2.2	T CELL ACTIVATION	3
1.2.3	T CELL SUBSETS AND T CELL MEMORY	5
1.3	IMMUNOLOGICAL TOLERANCE	6
<u>2</u>	<u>REGULATORY T CELLS</u>	<u>7</u>
2.1	TREG CELL DEVELOPMENT AND ACTIVATION	7
2.2	LINEAGE STABILITY VS PLASTICITY	10
<u>3</u>	<u>FOXP3</u>	<u>12</u>
3.1	FOXP3 GENE STRUCTURE AND EXPRESSION	12
3.2	FOXP3 PROTEIN STRUCTURE AND FUNCTION	14
3.3	FOXP3 ISOFORMS	14
3.4	FOXP3 MUTATIONS (IPEX)	16
<u>4</u>	<u>TREG CELL EFFECTOR FUNCTION</u>	<u>18</u>
4.1	CTLA-4 (CD152)	20
<u>5</u>	<u>TREG CELLS IN DISEASE</u>	<u>24</u>
<u>6</u>	<u>STUDY AIM</u>	<u>27</u>
<u>7</u>	<u>MATERIAL AND METHODS</u>	<u>29</u>
7.1	GENERATION OF CTLA4 ^{EX2FL/FL} FOXP3-CRE MOUSE	30
7.2	IN VITRO DCs SUPPRESSION AND T CELL PROLIFERATION	30
7.3	FOXP3 ISOFORM DETECTION	31
<u>8</u>	<u>RESULTS AND DISCUSSION</u>	<u>33</u>
8.1	PAPER I	33
8.2	PAPER II	35
8.3	PAPER III & IV	36
<u>9</u>	<u>CONCLUDING REMARKS</u>	<u>39</u>
<u>10</u>	<u>ACKNOWLEDGEMENTS</u>	<u>41</u>

LIST OF ABBREVIATIONS

AIRE	autoimmune regulator
APC	antigen presenting cells
CD	cluster of differentiation
CNS	conserved non-coding sequence
cTEC	cortical thymic epithelial cells
mTEC	medullary thymic epithelial cells
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
1/4 CTLA-4	CTLA-4 isoform lacking exon 2 and 3
flCTLA-4	full-length CTLA-4 isoform
liCTLA-4	ligand independent CTLA-4 isoform
sCTLA-4	soluble CTLA-4 isoform
DCs	dendritic cells
DN	double negative
DP	double positive
FOXP3	forkhead box protein 3
FOXP3 Δ 2	FOXP3 isoform lacking exon 2
FOXP3 Δ 2 Δ 7	FOXP3 isoform lacking exon 2 and 7
FOXP3 Δ 7	FOXP3 isoform lacking exon 7
Foxp3 δ 2 δ 7	FOXP3 isoform lacking exon 2 and 7 (mice)
FOXP3fl	full-length FOXP3 isoform
GARP	glycoprotein A repetitions predominant
GITR	glucocorticoid-induced TNFR-related protein
ICOS	inducible T-cell costimulator
IFN	interferon
IL	Interleukin
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked
JAK	Janus kinase
LRBA	Lipopolysaccharide-responsive and beige-like anchor protein
MHC	major histocompatibility complex
NK	natural killer cells
PAMPs	pathogen-associated molecules

PBMC	peripheral blood mononuclear cell
PD-1	programmed cell death protein 1
PD-L	programmed cell death-ligand
ROR	retinoic-acid-receptor-related orphan nuclear receptor
STAT	signal transducer and activator
PTA	peripheral tissue antigens
TCR	T cell receptor
TGF	transforming growth factor
T cell	T lymphocytes
CTL	CD8+ cytotoxic T cells
Th cells	CD4+ T helper cells
nT cells	naïve T cells
Teff cells	effector T cells
Treg cells	regulatory T-cells
tTreg cells	thymus-derived Treg cells
pTreg cells	periphery-derived Treg cells
rTreg cells	resting Treg cells
aTreg cells	activated Treg cells
TLR	toll-like receptor
TNFR	tumor necrosis factor receptor
TSDR	Treg-cell specific de-methylated region

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system consists of a network of organs, tissues and various types of specialized cells that together protect the body. This includes anatomical barriers like the skin and mucosal tissues, as our first line of defense. Additionally, the lymphatic system runs in parallel to the blood circulatory system and allows immune cells to traffic towards the destination where they perform their function. The lymphatic system functions both as a place for immune cell development, in the primary lymphoid organs (thymus and bone marrow) and as a site to orchestrate immune responses in secondary lymphoid organs (spleen and lymph nodes). Next to exposure to a variety of potential infectious agents including bacteria, viruses, and parasites on a daily basis. The immune system also needs to respond towards internal threats like damaged or mutated cells. The primary function of the immune system is to answer with an **immune response** to clear or control the spread of a threat. An immune response includes several phases: (I) antigen recognition, (II) clearance and/or limitation, (III) a return to immune homeostasis and (IV) formation of memory (Murphy and Weaver, 2017). In the context of this thesis the phases involve (I) activation of naïve T cells (section 1.2.2 and 2.1), (II) differentiation of Naïve T cells into effector T cells (section 1.2.3 and section 2.1), (III) limitation of T cell response (section 4) and (IV) T cell memory formation (section 1.2.3).

Not all antigens recognition provokes an immune response and in certain situations it could even have an adverse effect. Inappropriate immune response may lead to development of chronic inflammation, which in turn is associated with the increased risk of cancer development and autoimmune disorders (Doria et al., 2012; Grivennikov et al., 2010). Therefore, the immune system not only needs to be capable to distinguish between self and foreign antigens but also between dangerous and non-dangerous antigens. This concept that the immune system is able to identify dangerous from non-dangerous antigens is called immunological tolerance and will be discussed in section 1.3.

Throughout evolution, the immune system gained complexity through development of an innate and an adaptive arm, which today is present in higher vertebrates (Cooper and Alder, 2006; Janeway, 1992). The **innate immune system** comprises the rapidly responding part of the immune system and innate immune cells include granulocytes, natural killer (NK) cells and professional antigen presenting cells (APC) like macrophages and dendritic cells (DC). Innate immune cells recognize a broad range of pathogen-associated molecules (PAMPs) via conserved receptors such as toll-like receptors (TLRs). TLR activation leads to the secretion of cytokines and chemokines, i.e. molecules that mediate cell signaling, differentiation and migration. One of the key roles of the innate immune response involves the recruitment and activation of the adaptive immune system. DCs are often seen as a key link in connecting the innate and adaptive immune response due to their ability to process and present antigens and consequently induce T cell activation (see section 1.2.2) (Murphy and Weaver, 2017).

The **adaptive immune system** consists of two major classes of cells, namely B cells and T cells. Both cell types share the ability to clonally expand after recognition of their cognate antigen and further develop into memory cells. They both arise from the same multi-potent hematopoietic stem cells in the bone marrow but they differ in their site of development. B cells develop in the bone marrow, whereas T cell progenitors migrate to the thymus where they further differentiate into naïve T cells (see section 1.2.1 on T cell development) (Takaba and Takayanagi, 2017). The key feature of B cells is production and secretion of highly specific antibodies during humoral immune responses (Pieper et al., 2013). T cells are divided into two functional classes, namely CD4⁺ T cells and CD8⁺ T cells. CD4⁺ T cells support B cells during humoral response and CD8⁺ T cells can directly kill infected cells during cell mediated immune response (see section 1.2) (Murphy and Weaver, 2017).

One of the main differences between the innate and adaptive system is the development of immunological memory, which allows for a more efficient response upon re-infection. At the first encounter of a pathogen, the adaptive immune response requires up to 7 days to form an efficient response. During this phase B cells and T cells undergo a strict selection process resulting in highly specific cells for that particular triggering threat. After clearance a subset of the antigen-specific effector cells will differentiate in long living antigen-specific memory cells, which on a re-encounter will directly expand cell number thereby reducing the time to form an immune response. Immunological memory is a feature associated with adaptive immunity and the formation of long living antigen-specific memory cells (Murphy and Weaver, 2017; Natoli and Ostuni, 2019). In recent years the notion that memory is specific for adaptive immunity has been challenged as exemplified by memory NK cells (O'sullivan et al., 2015).

1.2 T CELLS

T cells recognize peptides (e.g. antigens) via expression of the highly specific T cell receptor (TCR) resulting in their activation (see section 1.2.2 on CD4⁺ T cell activation). Antigen presentation involves cells like APCs to process and present antigens bound to the major histocompatibility complex (MHC), forming a peptide/MHC complex. The MHC class I (MHC-I) molecule is expressed by most nucleated cells (including APCs) and in humans this class of molecules includes human leucocyte antigen A (HLA-A), HLA-B and HLA-C. The MHC class II (MHC-II) molecule is restricted to APCs including DCs, macrophages and B cells. In humans this group includes HLA-DM, HLA-DOA, HLA-DOB, HLA-DP, HLA-DQ and HLA-DR. The co-receptors CD4 and CD8 both stabilize the engagement of the TCR with MHC-II and MHC-I respectively. T cells are divided into the two major functional classes CD4⁺ helper T (Th) cells and CD8⁺ cytotoxic T cells (CTL) based on the expression of the co-receptors. CTLs can directly kill infected cells by secreting cytotoxic proteins like granzymes and perforin what makes them major players in cell-mediated immune response. In contrast, Th cells orchestrate immune responses by promoting the activation of various immune cell populations (Kumar et al., 2018; Murphy and Weaver, 2017).

1.2.1 Thymic development

T cells are derived from multipotent hematopoietic stem cells that leave the bone marrow and migrate to the thymus to form functional albeit not autoreactive T cells (Klein et al., 2014; Kurd and Robey, 2016; Takaba and Takayanagi, 2017) (see section 1.3 on Tolerance). In the thymic cortex double negative (DN) CD4⁻CD8⁻ thymocytes differentiate through several stages while keeping their DN profile and rearranging their **TCRs**. The TCR is a heterodimer protein consisting of either an alpha and beta chain ($\alpha\beta$ TCR) or gamma and delta chain ($\gamma\delta$ TCR). The TCR is generated through random somatic re-combination of the three gene segments encoding it. In principle TCR recombination can give rise to 10^{15} potential TCRs, however, in practice it is estimated that humans have 25 million unique TCRs that are meant to protect against an unknown number of adverse antigens (Arstila et al., 2000; Lythe et al., 2016). At this stage of their development the majority of thymocytes express an $\alpha\beta$ TCR and a subset of T cells will express a $\gamma\delta$ TCR. $\gamma\delta$ TCR expressing T cells can be mainly found in mucosal sites.

During TCR recombination DN thymocytes upregulate both CD4 and CD8 and become double positive (DP) CD4⁺CD8⁺ thymocytes that further undergo **positive selection**. DP thymocytes bind to self MHC proteins presented by cortical thymic epithelial cells (cTECs) survive the positive selection process where DP thymocytes with a too low affinity die from neglect. Depending on through which MHC molecule the T cells receive their signal they will either commit to become CD4⁺CD8⁻ or the CD4⁻CD8⁺ thymocytes. Positively selected cells will leave the cortex for the medulla to undergo negative selection. During TCR re-arrangement a subset of T cells are generated that can recognize self-antigens with a too high affinity. The aim of **negative selection** is to eliminate such autoreactive T cells and therefore negative selection will shape the TCR repertoire that will leave the thymus. Peripheral tissue antigens (PTA) are presented on DCs and medullary thymic epithelial cells (mTECs) The transcription factor AIRE (autoimmune regulator) plays a critical role in mTECs differentiation and drives the expression of PTAs (Nishikawa et al., 2014; Passos et al., 2018). However, a subset of T cells with relatively high affinity to self-antigens will pass negative selection and differentiate into regulatory T cells (see section 2.1 on Treg cells development).

1.2.2 T cell activation

After leaving the thymus naïve T cells circulate the body searching for their TCR specific antigen. Unlike some innate immune cells, T cells cannot directly recognize pathogens but rely on antigen presentation via the peptide/MHC complex for activation. The adaptive immune response is initiated in secondary lymphoid tissues, e.g. lymph nodes, which includes the priming of naïve T cells. In short, naïve T cells express the chemokine receptor CCR7 which promotes their migration via the blood system to the T cell zones of lymph nodes where CCL21, a ligand for CCR7 is produced. DCs from the periphery enter the lymph node where they encounter naïve T cells and present their antigens via the peptide/MHC complex, which can result in T cell activation. The classical model of T cell activation involves three signals: (I) antigen recognition via TCR engagement, (II) co-stimulatory signaling via CD28 engagement

with CD80 and/or CD86 and (III) cytokine signaling through their receptor promoting differentiation into the effector cell lineage (see section 1.2.3).

T cell activation starts with **TCR engagement** of the peptide/MHC complex. CD8⁺ CTLs normally recognize endogenous peptides presented on MHC-I. However, during the activation process CTLs depend on recognizing exogenous peptides presented on MHC-I by professional APCs, in a process known as cross-presentation. Since most nucleated cells express MHC-I molecules it will later allow the CTLs to directly recognize and kill infected cells. CD4⁺ T cells instead rely on antigen presentation of peptides presented in the context of MHC-II. In most cases TCR stimulation alone would not be sufficient to initiate activation and T cells lacking additional signals would either enter the stage of unresponsiveness, i.e. anergy, or go into apoptosis (Kündig et al., 1996). This is exemplified by CD28 knock-out mice that exhibit an impaired immune response due to a lack of Th cell activity, however, long term TCR signaling might be CD28 independent (Shahinian et al., 1993).

APCs like DCs express **co-stimulatory** molecules such as CD80 and/or CD86. Naïve DCs mainly express CD86 but upon activation via TLR engagement, DCs start to upregulate both molecules that in turn bind CD28 on T-cells providing a stronger second signal (Sansom et al., 2003). Blocking of this second signal is a well-studied process in immune regulation and opens possibilities for clinical intervention.

The cytokine **IL-2** is primarily produced by activated CD4⁺ T cells and it is often referred to as a survival factor of T cells. It binds to the IL-2 receptor resulting in intracellular signaling that promotes T cell proliferation. The **IL-2 receptor (IL-2R)** is a heterotrimeric protein that depending on the receptor chains forming it, has a low, intermediate or high binding affinity for IL-2. The three receptor chains that exist are CD25, the interleukin-2 receptor chain α (IL-2R α), IL-2R β (CD122) and IL-2R γ_c (CD132). IL-2R β/γ_c together have an intermediate affinity receptor for IL-2 but both protein chains are not restricted to IL-2R formation. IL-2R γ_c is also part of the IL-7 receptor, whereas both IL-2R β/γ_c are part of the IL-15 receptor. IL-2R α alone has low affinity but in combination with IL-2R β/γ_c forms the high affinity IL-2R. CD25 by itself is not involved in IL-2 signaling but supports binding of IL-2 where IL-2R β/γ_c signaling results in activation of pathways involved in cell differentiation, proliferation, survival and activation-induced cell death (Fontenot et al., 2005). IL-2 signaling also stimulates the upregulation of other cytokine receptor chains involved in differentiation of naïve T cells into a different T cell subset. This includes IL-12R β (CD212) and IL-4R α (CD124) (Liao et al., 2008) and suppressed IL-7R- α (CD127) expression (see section 1.2.3 on T cell subsets). Additionally, IL-2 acts in a positive feedback loop by stimulating the upregulation of IL-2R α and IL-2R β (Liao et al., 2011). Taken IL-2 signaling plays a central role in T cell development as well as maintenance and is therefore often referred to as T cell survival factor.

1.2.3 T cell subsets and T cell memory

Antigen recognition and co-stimulation alone can activate naïve T cells and induce cell proliferation, however T cell differentiation into T effector (Teff) cells requires additional signals provided by cytokines. Depending on the combination of cytokines provided by T cells themselves, APCs and other immune cells CD4⁺ T cells can differentiate into several effector Th cell subsets.

Upon activation naïve CD8⁺ T cells develop their cytotoxic phenotype that provides them with the ability to directly kill infected cells. Naïve CD4⁺ T cells differentiate into a variety of functional different **T helper (Th) subsets**, depending on the environmental signals they receive during T cell activation. The best characterized Th subsets are Th1, Th2, Th17 and follicular helper cells. Beside differentiation into Teff cells, naïve T cells can differentiate into Treg cells in the presence of TGF- β and IL-2 (discussed in detail in section 2.2).

In the presence of the cytokines IL-12, IL-18 and IFN γ as well as signal transducer and activator 4 (STAT4) signaling T cells differentiate into **Th1**. The transcription factor T-bet is the lineage defining transcription factor of Th1 cells and Th1 immune responses are aimed towards intracellular pathogens (e.g. viruses) and if not controlled properly could result in inflammatory diseases. **Th2** cells differentiate in the presence of IL-4 and STAT5A signaling and IL-2 signaling can enhance IL-4 transcription (Liao et al., 2008). Furthermore, transcription factor GATA-3 plays a central role in maintaining Th2 lineage. Th2 cells play a crucial role in facilitating an immune response against extracellular pathogens (e.g. parasites) but are also associated with allergic inflammation. Th2 signature cytokines include IL-4, IL-5 and IL-13. The lineage defining transcription factor for **Th17** is retinoic-acid-receptor-related orphan nuclear receptor γ t (ROR γ t). Cytokines involved in Th17 differentiation include TGF- β , IL-21 and IL-23 and intracellular signaling pathways via STAT6 and STAT3. Upon activation Th17 produce cytokines including IL-17A, IL-17F and IL-22 that support anti-fungal and anti-bacterial immune responses. Miss-managed Th17 responses are associated with autoimmune disorders.

After activation CD4⁺ Teff cells undergo rapid proliferation during an expansion phase followed by a functional phase where they exert their functions. When the initiating factors are eliminated the Teff cell pool contracts and a majority of Teff cells undergo apoptosis. The CD4⁺ T cell **memory** pool is formed during the contraction phase from Teff cells that avoid apoptosis. Depending on where these cells persist they are referred to as central memory T cells (secondary lymph organs), effector memory T cells (periphery) or tissue-resident memory T cells.

With the advent of fate-mapping animal models and high-throughput sequencing technologies like single cell RNA sequencing it became more and more evident that T cells display a high plasticity and the concept of true lineage commitment is challenged.

1.3 IMMUNOLOGICAL TOLERANCE

Immunological tolerance is defined as the unresponsiveness of the immune system to antigens that were identified as non-dangerous. Depending on the anatomical side where tolerance is established, we speak of central or peripheral tolerance. Several key regulators for establishing and maintaining tolerance were identified, including FOXP3, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and AIRE (Anderson and Su, 2016; Bluestone, 1997; Gambineri et al., 2018; Lu et al., 2017).

At first sight a decreased immune response, or total lack of it, can appear counterintuitive. However, the importance of immunological tolerance can be seen when the immune system fails to develop tolerance towards certain antigens. Break down of immunological tolerance leads to chronic inflammatory responses such as seen in patients with autoimmune diseases. Additionally, tolerance against non-harmful environmental and food antigens are critical to prevent allergic reactions.

Central tolerance develops in primary lymph organs. A crucial step in establishing central tolerance is the capacity of the immune system to identify self-antigens followed by deletion of strongly autoreactive lymphocytes in the thymus and bone marrow during T cell and B cell development respectively. Early studies on thymic function revealed that after neonatal thymectomy mice develop autoimmune diseases if conducted 3 days after birth. It confirmed that cells within the thymus play an important role in immune homeostasis (Gershon and Kondo, 1970; Nishizuka and Sakakura, 1969). Later it was found that during T cell development the expression of AIRE in mTEC plays a crucial role by regulating the expression of tissue specific antigens in the thymus and consequently shaping central tolerance during negative selection (see section on thymic development) (Anderson and Su, 2016). In humans, mutations in AIRE causes the development of an autoimmune phenotype called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (Aaltonen et al., 1997).

Peripheral tolerance establishes through cells in secondary lymphoid organs, and other immunological relevant tissues such as mucosal surfaces in the lung and intestine. There are several ways of achieving tolerance in the periphery including induction of anergy and cells with immunosuppressive function. Anergy is defined as unresponsiveness to antigenic stimulation and occurs when T cells recognize their antigen in the absence of costimulatory molecules. Furthermore, in both central and peripheral tolerance a subset of CD4⁺ T cells, called Treg cells, is critical. These cells express two crucial regulators in immunological tolerance, the transcription factor FOXP3 and the co-inhibitory receptor CTLA-4 (Taams et al., 2002) (see section 3 on FOXP3 and section 4.1 on CTLA-4). Both, FOXP3 and CTLA-4 and their functional role in Treg cells are topic of this thesis and will be discussed in greater detail below.

2 REGULATORY T CELLS

Treg cells have historically been a controversial topic that fell out of favor in the late 1980s (Möller, 1988). Treg cells then made a reappearance in the mid 1990s when Sakaguchi *et al.* (1995) demonstrated that CD4⁺CD25⁺ T cells exhibit a strong suppressive phenotype (Sakaguchi *et al.*, 1995) and in the early 21st century the transcription factor FOXP3 (transcription factor forkhead box P3) was identified as the lineage defining transcription factor for cell development and stability (Fontenot *et al.*, 2003; Hori *et al.*, 2003). Treg cells are a subset of CD4⁺ T cells and in humans the frequency of peripheral blood Treg cells varies between 5-10 percent out of all CD4⁺ T cells (Dieckmann *et al.*, 2001).

In the last two decades several markers were proposed to define Treg cells and these markers vary depending on species. The first described marker, **CD25**, is highly expressed on Treg cells, both in human and mice, and is even further upregulated upon TCR stimulation (Baecher-Allan *et al.*, 2001; Dieckmann *et al.*, 2001; Sakaguchi *et al.*, 1995). In mice, CD25 expression is mainly restricted to the Treg cell pool and Treg cells are therefore commonly defined as CD4⁺CD25^{hi}. Liu *et al.* showed that **CD127**, the interleukin-7 receptor α chain (IL-7R- α) is negatively associated on human Treg cells (Liu *et al.*, 2006). Therefore, human Treg cells are often referred to as CD4⁺CD25^{hi}CD127^{lo} cells. If restricted to extracellular markers e.g. to perform *in vitro* studies on viable cells, the combination of these three markers is used to define Treg cells. Otherwise, **FOXP3** expression is used to define Treg cells. In several studies it has been shown that FOXP3 expression is highly restricted to the Treg cell pool in mice (Fontenot *et al.*, 2005a) but in contrast, human Treg can transiently express FOXP3 upon activation *in vitro* (Walker *et al.*, 2003). It was shown that the Treg cell pool is a heterogeneous population and can be further distinguished based on its developmental origin activation status and functional activity (see sections below) (Kanamori *et al.*, 2016; Li *et al.*, 2015; Richards *et al.*, 2015).

Furthermore, Treg cells are also characterized by their expression of markers related to their functional properties which include CTLA-4, glycoprotein A repetitions predominant (GARP), glucocorticoid-induced TNFR-related protein (GITR), inducible T-cell costimulator (ICOS), CD37 and CD39 and will be discussed in section 4. Furthermore, markers used to determine Treg subpopulations based on activation include CD45RA, CD45RO, CD44, CD62L and CD69 and will be discussed in section 2.1 (Rosenblum *et al.*, 2016; Sakaguchi *et al.*, 2010).

2.1 TREG CELL DEVELOPMENT AND ACTIVATION

Treg cells can develop via two distinct routes, either in the thymus alongside with naïve CD4⁺ T cells or in the periphery where they are induced from naïve CD4⁺ T cells. These two primary Treg populations not only differ in their anatomical site of origin but might also play different roles in maintaining immune hemostasis and upholding tolerance. Where thymus derived Treg (tTreg) cells are described to be more involved in limiting autoimmune responses, peripheral induced Treg (pTreg) cells are described to be functional more involved at tissue

sites exposed to environmental boundaries agents like mucosal tissues (Arpaia et al., 2015; Josefowicz et al., 2012; Taams et al., 2002). However, even though different environmental signals can induce Treg cell differentiation, in both cases the upregulation and stable expression of FOXP3 is seen as the key indicator for Treg cell development (see section 3.1) (van Nieuwenhuijze and Liston, 2015).

With an increase in research on Treg cell biology, several molecules were introduced as potential markers to distinguish the tTreg and pTreg populations. First **helios** and later **neuropilin-1** were postulated to be expressed by tTreg cells only (Thornton et al., 2010; Weiss et al., 2012; Yadav et al., 2012). More recent studies challenged this concept showing that that helios as well as neuropilin-1 can be upregulated upon Treg cell activation. (Gottschalk et al., 2012; Himmel et al., 2013; Szurek et al., 2015). So far, there is no consensus on what combination of markers can be used to define tTreg cell and pTreg cells populations.

Thymus derived Treg (tTreg) cells split from the conventional T cell lineage during negative selection and follow a two-step developmental program (Burchill et al., 2008; Lio and Hsieh, 2008). When TCR reactivity is tested during negative selection, cells with a higher affinity to self-antigens than Tn develop towards the Treg cell lineage (Jordan et al., 2001). Except for a small overlap, the TCR repertoire of Treg cells differs from Tn cells and also has greater diversity (Hsieh et al., 2004; Pacholczyk and Kern, 2008; Pacholczyk et al., 2006). However, the exact selection criteria on TCR reactivity during negative selection is still poorly understood.

Following a two-step developmental program in the thymus, cell differentiation starts with TCR stimulation together with CD28 engagement what in turn results in the upregulation of proteins supporting Treg cell differentiation including CD25, GITR, CD134 and tumor necrosis factor receptor 2 (TNFR2) (Mahmud et al., 2014; Tai et al., 2005; Vang et al., 2010). The following step is independent of TCR signaling and instead depends on cytokine signaling. IL-2 is important for tTreg cell differentiation. Both, *il2* deficient and *il2ra* deficient mice models showed the loss of IL-2 signaling reduces tTreg cell output. However, several studies indicated that IL-2 signaling can be partly compensated for via IL-15 and IL-7 signaling. Both signaling pathways involve receptor complexes that share protein chains with the IL-2R (Bayer et al., 2008; Fontenot et al., 2005a). Additionally, it was shown that the transcription factor STAT5 plays an important role during the second phase of tTreg development (Burchill et al., 2007; Yao et al., 2007). In a recently published study by *Owen et al.* (2019), the authors postulate that tTreg cells can develop via two different developmental programs. In the study two distinct Treg precursors were identified, CD25⁺FOXP3⁻ and CD25⁻FOXP3^{lo}, that both can develop into mature Treg cell *in vivo* and *in vitro*. These progenitors differ among other things in their affinity to self-peptides, cytokine responsiveness and function (Owen et al., 2019). Nevertheless, the exact mechanism during negative selection of self-reactive cells leading to the development of tTreg cells rather than apoptosis is still not fully understood (Richards et al., 2015).

TGF- β can stimulate the expression of FOXP3 via induction of proteins that in turn promote FOXP3 transcription (see section 3.1). TGF- β was shown to support the differentiation of peripheral Treg (**pTreg**) cells from naïve CD4⁺FOXP3⁻ T cells. Naïve T cells that would go into anergy due to an ongoing low TCR stimulation can commit to the Treg cell lineage. This stands in contrast to tTreg cells, whose differentiation relies on high TCR affinity and the TCR remount of pTreg cells differs and for example, have TCRs related to the exposure to environmental surfaces like commensal bacteria (Round and Mazmanian, 2010). Furthermore, experiments to generate induced Treg (**iTreg**) cells *in vitro* showed the combination of TGF- β and IL-2 stimulated FOXP3 expression. However, iTreg cells display a problem maintaining their FOXP3 expression (Kanamori et al., 2016).

Similar to effector T cells, TCR stimulation is needed but not sufficient for **Treg cell activation**. An important additional signal is provided by the co-stimulatory receptor CD28 on T cells that binds CD80 and CD86 expressed by antigen presenting cells (APC) like dendritic cells (DCs). Using *Cd28* deficient mice models it was shown that CD28 signaling supports tTreg development and upregulation of GITR and CTLA-4, but not complete loss of tTreg (Mahmud et al., 2014; Tai et al., 2005; Vang et al., 2010). Furthermore, it was shown that CD28 or CD80/CD86 deficiency leads to a reduction in Treg cell numbers that is even more pronounced than for Teff cells. Specific CD28 deletion in FoxP3⁺ Treg cells leads to the development of lymphoproliferative diseases (Zhang et al., 2013).

In effector T cells, CD28 signaling induces IL-2 production but unlike Teff cells, Treg cells cannot produce IL-2 themselves (Ono et al., 2007). IL-2, although not absolutely crucial for Treg cell development, is important for Treg cell differentiation and maintenance. Germline deficient mice either in CD25 or IL-2 show reduced Treg cell numbers (Fontenot et al., 2005a). In contrast, mice intravenously treated with IL-2 had increased Treg cell numbers. Subsequently, a CD3/CD28 stimulation in combination with recombinant IL-2 is used for Treg cell activation in *in vitro* settings or for *ex vivo* expansion studies (Hoffmann et al., 2004).

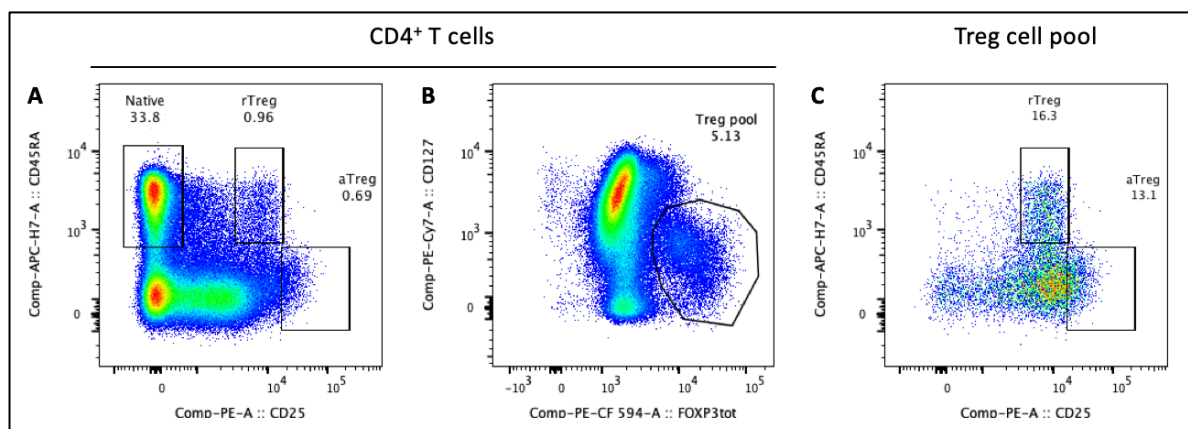


Figure 1: Flow cytometry staining of human PBMCs. (A) The combination of CD45RA and CD25 expression is used to define Treg sub-populations. Treg cells in general are higher in their CD25 expression than naïve T cells. (B) To define rTreg and aTreg populations first the Treg cells pool is defined as FOXP3^{hi}CD127^{lo} cells (C) followed by gating of CD45RA⁺FOXP3^{lo}CD25⁺ rTreg and CD45RA⁺FOXP3^{hi}CD25⁺ aTreg cells.

Miyara et al. (2009) showed the Treg cell pool is not homogeneous rather these cells differ phenotypically and functionally. They defined three distinct FOXP3 expressing CD4⁺ T cells groups namely, CD45RA⁺FOXP3^{lo}CD25⁺⁺ resting Treg (**rTreg**) cells, CD45RA⁺FOXP3^{hi}CD25⁺⁺⁺ activated Treg (**aTreg**) cells and CD45RA⁺FOXP3^{lo}CD25⁺⁺ non-Treg cells (see Figure 1) (Miyara et al., 2009). CD45RA together with CD45RO are used to distinguish resting from activated Treg cells since both proteins are not expressed simultaneously (Booth et al., 2010). **rTreg** cells exhibit suppressive capacities and are able to maintain their FOXP3 expression *in vitro* but in general display a lower expression of functional markers like CTLA-4. Furthermore, they are shown to be negative for Ki67 expression and start to proliferate in *in vitro* cultures upon activation. On the other hand, **aTreg** cell express higher levels of functional markers and are Ki67⁺ but do not proliferate well *in vitro* and tend to die. Recently, higher CD15s expression was introduced as an additional marker to better define aTreg cell populations (Miyara et al., 2015).

As mentioned earlier, Teff cells can differentiate into memory T cells. Several groups postulated the idea of regulatory T cell memory. However, to classify memory Treg cells based on phenotypical and functional markers is challenging. Phenotypically they show a great overlap with aTreg cells such as expression of functional markers like CTLA-4 and ICOS. Furthermore, it is yet to be seen whether Treg cells survive prolonged periods without TCR stimulation which would be a characteristic of memory cells (Rosenblum et al., 2016).

Altogether Treg cell development and activation is a multistep process differing between pTreg and tTreg cells (Richards et al., 2015). Regardless of which signal cascade stimulates Treg cells generation the induction of FoxP3 is crucial.

2.2 LINEAGE STABILITY VS PLASTICITY

The improvement of omics and other technologies which helps us to study rare cell populations has contributed to our understanding of Treg cell biology and it is becoming increasingly clear that Treg cells represent a heterogeneous cell population. Several recent studies suggest that Treg cells display a higher plasticity than initially thought but it is still under debate if this might include either lineage plasticity or functional plasticity or even both.

FOXP3 stability is in the center of this discussion since it was seen that FOXP3 is a key regulator in Treg cell biology and induction and maintaining of FOXP3 expression is regulated separately (Zheng et al., 2010). It was shown that Treg cells can maintain their suppressive phenotype and FOXP3 expression while proliferating and expanding. Furthermore, studies on epigenetic control linked the de-methylation of the FOXP3 promotor region with a stable expression of FOXP3 in Treg cells (Floess et al., 2007; Kim and Leonard, 2007). Rubtsov et al. (2010) studied Treg cell plasticity using a Treg cell fate mapping mouse model (*Foxp3*^{GFP-Cre-ERT2} knock-in *ROSA26*^{YFP} mice) and found that during both homeostasis and inflammation Treg cells remain stable indicating that tTreg cells might be more stable than pTreg cells (Rubtsov et al., 2010).

Furthermore, it was shown that pTreg cells might be epigenetically less stable in respect to their FOXP3 expression (Polansky et al., 2008).

In contrast, in several studies it was shown that Treg cells may lose their FOXP3 expression in pro-inflammatory environments and develop a more effector Th cell phenotype in response to certain environmental signals. It was reported that Treg cells can start to produce effector cytokines like IL-2 and IL-17 (Duarte et al., 2009; Yang et al., 2008). Furthermore, Teff cells can transiently express FOXP3 (see section 3) and recent studies showed FOXP3 expressing Teff cells can potentially differentiate into Th17 cells in the presence of IL-1 β (Mailer et al., 2015). Treg cell plasticity towards the Th17 phenotype was also described in rheumatoid arthritis mice models where the authors showed that Th17 can develop from FOXP3⁺CD25^{lo} cells (Komatsu et al., 2014). It was also shown that Treg cells differentiate at different anatomical sites. The existence of tissue specific Treg cells indicates Treg cells can be influenced by their environment. However, the lack of markers to distinguish tTreg and pTreg cell populations makes it challenging to address the question if observed lineage plasticity is due to the pTreg cells (Polansky et al., 2008).

3 FOXP3

FOXP3 is a forkhead/winged-helix transcription factor that confers its function by binding DNA as part of a multi-protein complex to activate or repress transcription. FOXP3 was linked to immune regulation in the early 2000s when it became evident that a frame-shift mutation of FOXP3 was responsible for the lymphoproliferative disease of scurfy mice (Bennett et al., 2001; Brunkow et al., 2001). In 2003 three research groups demonstrated FOXP3 was the master regulator for Treg cell development. More recent studies suggest FOXP3 expression does not automatically indicate functional Treg cells (Tran et al., 2007). Human effector T cells can transiently express FOXP3 without being immune suppressive (Allan et al., 2007; Miyara et al., 2009).

3.1 FOXP3 GENE STRUCTURE AND EXPRESSION

In both, human and mice, the *FOXP3* gene is located on the X-chromosome and consists of eleven coding exons (1 - 11) and three non-coding exons (-2a to -1). The eleven coding exons encode four protein domains. Exon 1 to 4 encode the repressor domain, exons 5 and part of 6 the zinc finger domain, the other part of exon 6 together with exon 7 encode for the leucine zipper and exon 9 to part of 11 the forkhead (FKH) domain (Figure 2). Exons 2 and 7 are both simultaneously and separately alternatively spliced (see section 3.3 on FOXP3 isoforms). FOXP3 expression is regulated by the *FOXP3* promoter together with three enhancers (conserved non-coding sequences, CNS). FOXP3 expression is strongly related to Treg cell differentiation and activation and relies on similar pathways namely TCR signaling, co-stimulation and cytokine signaling (Figure 3) (Huehn and Beyer, 2015; Huehn et al., 2009; Kitagawa et al., 2015b; 2015a).

Besides the FOXP3 promotor, the three non-coding sequence regions CNS1, 2 and 3, all interact with different proteins and are involved both in initiation and maintenance of FOXP3 expression but differ in respect to which phases of Treg cell development they are involved in. **CNS3** was described to initiate FOXP3 expression and binding of c-Rel to CSN3 was described to be crucial for tTreg development (Ruan et al., 2009). **CNS2** is a CpG-rich sequence enhancing and stabilizing the expression of FOXP3 (Polansky et al., 2008). It is the site of epigenetic control of FOXP3 expression and de-methylation stabilizes FOXP3 expression. CNS2 is also referred to as Treg-cell specific de-methylated region (TSDR) and in Teff cells this specific region is methylated. Furthermore, several transcription factor binding sites were described in CNS2, including GATA3, ETS1 and FOXP3 itself. The deletion of CNS2 results in a gradual loss of FoxP3 in mice (Ogawa et al., 2014; Zheng et al., 2010). FOXP3 expression regulation via **CSN1** is associated with pTreg development. **TGF- β** signaling promotes FOXP3 expression by upregulating the transcription factors Smad3 which acts via binding to the CNS1 region (Schlenner et al., 2012; Zheng et al., 2010).

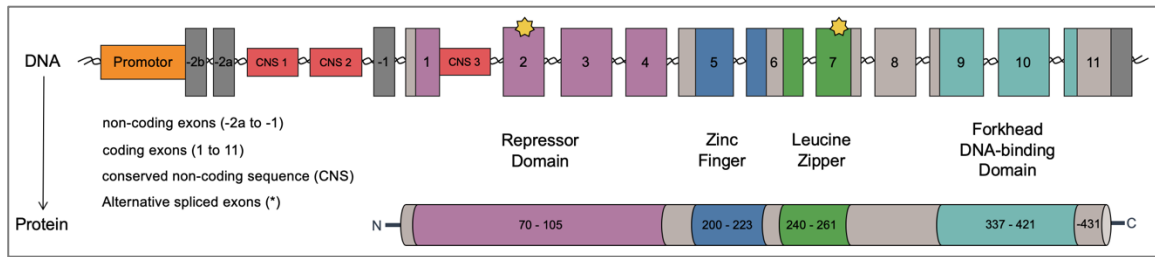


Figure 2: Schematic representation of the FOXP3 gene and protein. FOXP3 gene consist of three non-coding exons (-2a, -2b and -1) has three conserved no-coding sequences (CNS1, CNS2 and CNS3) and eleven exons (1 - 11). Exon 1 to 11 encodes for the four functional regions, three repressor domain, zinc finger, leucine zipper and the Forkhead DNA-binding domain.

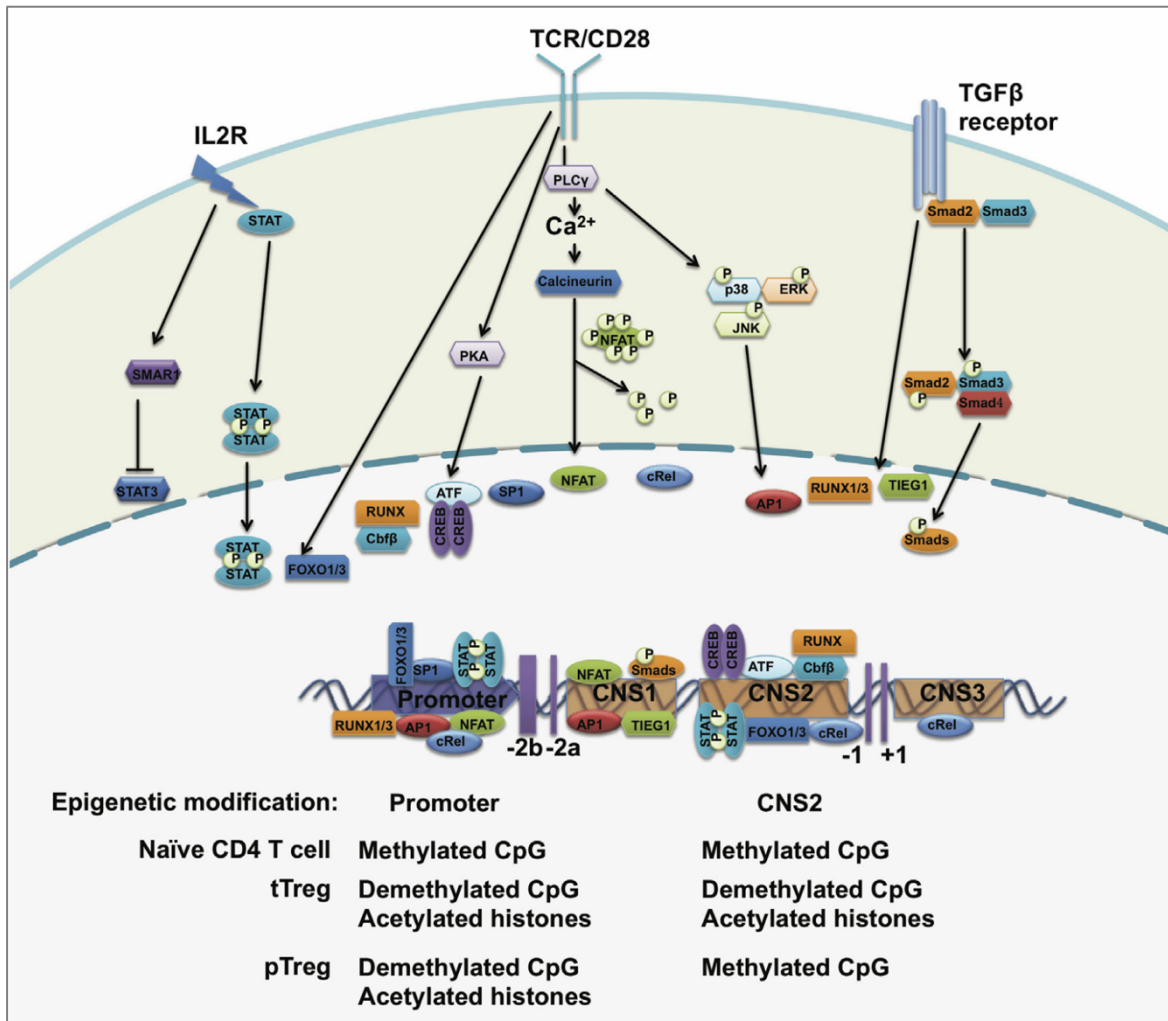


Figure 3: FOXP3 expression in CD4+ T cells. A combination of TCR, co-stimulatory and cytokine signaling induced FOXP3 expression. Both the FOXP3 promotor and CNS regulate transcription via interacting with different proteins. Adapted from (Nie et al., 2015)

Furthermore, **TCR signaling** and co-stimulatory signaling trough **CD28** both upregulate FOXP3 expression (Salomon et al., 2000; Tai et al., 2005). TCR signaling results in binding of NFAT (nuclear factor of activated T cells) and AP1 (activator protein 1) which both induce FOXP3 expression by binding to its promotor and CNS1 (Mantel et al., 2006). Interestingly, expression of NFAT in conjunction with RUNX1 and FOXP3 leads to a reduction in Treg function (Ono et

al., 2007; Wu et al., 2006). In the thymus CD28 stimulation was seen to induce FOXP3 expression (Tai et al., 2005) but strong CD28 signaling in the periphery was shown to negatively regulate FOXP3 expression. The **IL-2** signaling cascade involves Janus kinase 1 (JAK1) and JAK3 and the signal STAT5. STAT5 in turns binds the FOXP3 promotor as well as CNS2 leading to increased FOXP3 expression and stabilization.

3.2 FOXP3 PROTEIN STRUCTURE AND FUNCTION

FOXP3 consists of four domains, namely the repressor domain at the N-terminal end of the protein, followed by the zinc finger domain, the Leucine zipper domain and the Forkhead DNA binding (FKH) domain at the C-terminal end (Figure 2). Treg cells express a distinct set of differentially regulated genes in comparison to effector T cells. It has been shown that FOXP3 is able to control expression of around half of these genes and FOXP3 is described to work both as a suppressor and activator of mRNA translation. Genome-wide studies revealed that FOXP3 in collaboration with other proteins control over 700 genes but only a fraction of these genes is directly controlled by FOXP3 binding. (Marson et al., 2007; Zheng et al., 2007). So far, over 360 proteins, including other transcription factors, were reported to interact with FOXP3 and act in protein complexes to regulate transcription (Hori, 2012; Lozano et al., 2013; Rudra et al., 2012; Sadlon et al., 2010).

The N-terminal **repressor domain** contributes to the suppressive activity of Treg cells. This FOXP3 domain mediates gene expression by interacting with transcriptional activators and repressors. For example, FOXP3 interacts with members of the NF- κ B family proteins known to be responsible for a variety of pro-inflammatory cytokine up-regulation (Bettelli et al., 2005; Deng et al., 2012; Lopes et al., 2006). Furthermore, it was reported transcription factors of the ROR family, ROR α and ROR γ t, bind to motifs expressed by exon 2 leading to suppression of Th17 differentiation (Ichiyama et al., 2008; Zhou et al., 2008). Also, STAT3 interacts with FOXP3 by binding to the repressor domain. The function of the **zinc finger domain** remains unclear. FOXP3 requires dimerization via the **leucine zipper domain** to bind to DNA (Chae et al., 2006). Beside binding to itself, FOXP3 also binds other proteins like FOXO1 via the leucine zipper domain to regulate additional aspects of FOXP3 functions. Treg cells is regulated via this domain. FOXP3 binds to DNA via its **FKH domain** via the GTAAACA motive (Koh et al., 2009). Furthermore, some of Treg cells most important regulatory mechanism are controls via this domain. For example, FOXP3 DNA binding is associated with the repression of IL-2 and the expression of CD25 and CTLA-4 (Hill et al., 2007; Marson et al., 2007; Ono et al., 2007).

3.3 FOXP3 ISOFORMS

The *foxp3* gene consists of eleven coding exons and when fully transcribed form the full-length isoform of FOXP3, FOXP3fl. In human, three additional isoforms that are the result of alternative splicing of exon 2 and/or exon 7 were identified. Alternative splicing of the two

exons give rise to the isoforms lacking either exon 2 (FOXP3 Δ 2) or exon 7 (FOXP3 Δ 7). Additionally, alternative splicing of both regions results in the so called FOXP3 Δ 2 Δ 7 (Kaur et al., 2010; Mailer et al., 2009; Smith et al., 2006; Walker et al., 2003). FOXP3 isoforms differ in their molecular weight, where FOXP3fl has the highest weight with 47 kDa, followed by FOXP3 Δ 7 with 44 kDa, FOXP3 Δ 2 with 43 kDa, and FOXP3 Δ 2 Δ 7 with 40 kDa. The molecular weight of both FOXP3 Δ 2 and FOXP3 Δ 7 is challenging to distinguish using techniques like western blot.

FOXP3fl and **FOXP3 Δ 2** are the predominant FOXP3 isoforms and both are simultaneously expressed by Treg cells and contribute to the suppressive function of Treg cells. Exon 2 encodes a part of the repressor domain that, as mentioned above, is involved in the interaction with protein binding partners. Therefore, it can be assumed that alternative splicing of this region has an effect on FOXP3 to form protein complexes. For example, it was shown that exon 2 encodes for a binding site for transcription factors of the ROR family and as a result suppress IL-17A (Du et al., 2008). In turn, ROR γ t is a regulator of Th17 differentiation indicating the importance of the repressor domain in balancing differentiation between Treg cells and Th17 cells.

Our group has recently described a role for FOXP3 isoforms in regulating T cell differentiation between Treg cells and Th17 cells. In brief, IL-1 β favors Th17 differentiation *in vitro* by promoting **FOXP3 Δ 2 Δ 7**. Also, we observed a correlation between FOXP3 lacking exon 7 variant expression and clinical score in biopsies of Crohn's disease patients (Mailer et al., 2015). In contrast to Foxp3fl and Foxp3 Δ 2, Foxp3 Δ 2 Δ 7 does not contribute to the suppressive ability of Treg cells. Exon 7 encodes part of the leucine zipper and deletion of exon 7 is likely to interfere with the dimerization of FOXP3. In a study published 2015, we generated mice expressing the murine equivalent of Foxp3 lacking exon 2 and 7 (Foxp3 δ 2 δ 7) only. Males developed a phenotype similar to scurfy mice. This suggests that Foxp3 δ 2 δ 7 does not confer a suppressive function of Treg cells *in vivo* (Joly et al., 2015). Expression of **FOXP3 Δ 7** was detected in human T cells using qPCR but so far it is unclear how its function differs from the other three isoforms (Kaur et al., 2010) Since FOXP3 Δ 7 is lacking part of the leucine zipper domain that takes part of the dimerization of FOXP3 protein it might lack the ability to support Treg cell suppressive function. This is supported by the finding of an IPEX patient with a mutation that excludes exon 7 from the protein.

The exact signals activating alternative splicing and the mechanism by which it is regulated remain poorly understood. Also, little is known regarding the differences in protein function and capacity of interaction with co-factors of the different isoforms. Studying FOXP3 isoforms can be challenging with commonly used methods in immunology research that depend on the use of antibodies, including flow cytometry and SDS-PAGE.

3.4 FOXP3 MUTATIONS (IPEX)

Both, IPEX syndrome in humans and the scurfy phenotype in mice are the result of mutations in the *FoxP3* gene. **Scurfy mice** were first described in the late 1950^s and characterized as a spontaneous, recessive X-linked mutation. First symptoms in affected mice start to show at around 2 weeks of age as reddening and swelling of outer genitalia shortly after followed by ruffled, scaly skin of e.g. ears, tail and eyelids. In general, these animals are smaller, display a hunched posture and the majority of scurfy mice died at around 3 weeks of age (before 40 days). In the early 1990s the immune phenotype of scurfy mice was characterized as a lymphoproliferative disease. Animals have an enlarged liver, spleen and LNs including immune cell infiltration. Additionally, scurfy mice have elevated IgG levels (Godfrey et al., 1991a; 1991b). The scurfy phenotype was later linked to a 2-base pair insertion in exon 8 of the *Foxp3* gene resulting in a loss-of-function mutation in the FOXP3 protein (Brunkow et al., 2001).

In human, mutations on any of the regions of the *FOXP3* gene is linked to the development of **IPEX** (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), a syndrome characterized by the development of multiple autoimmune disorders with an onset typically within the first few months of life (Bennett et al., 2001; Chatila et al., 2000). IPEX is a rare X-linked disease with an estimated prevalence of 1 in 1.6×10^6 . Onset and severity vary between affected individuals but common clinical phenotypes include autoimmune enteropathy, dermatitis and poly-endocrinopathy including type 1 diabetes. Table 1 summarized most common symptoms of IPEX patients (Gambineri et al., 2018; Wildin et al., 2001). More recent studies also indicate a correlation between IPEX and miscarriage (Reichert et al., 2016; Xavier-da-Silva et al., 2015). Treatment of IPEX depends on the individual manifestation and ranges from symptom specific treatments (e.g. insulin in case of type 1 diabetes) over immune suppressive drugs, to bone marrow transplantation in severe cases (Gambineri et al., 2018).

Table 1: Overview of IPEX symptoms. IPEX is an autoimmune phenotype in humans carrying mutations in the *Foxp3* gene. Clinical manifestation varies between patients and this table summarized frequencies of disease associated symptoms (Gambineri et al., 2018).

Symptom	Frequency	
Enteropathy	97%	Watery diarrhea (minority blood/mucus), Atrophy on small bowel (45%)
	36%	Severe food allergy
Skin Disease	89%	Eczema and/or exfoliative (85%), Erythroderma (8%), childhood alopecia (11%)
Endocrinopathy	65%	Type I diabetes mellitus (49%, mean onset 27 month), hypo/hyperthyroidism (26%), adrenal insufficiency
Serious Infections	47%	sepsis, meningitis, peritonitis, pneumonia
		Staphylococcus (23%), Cytomegalovirus (10%), Candida (19%)
Hematologic disease	42%	Anemia (33%), neutropenia (13%), thrombocytopenia (22%), Organ /non-organ specific autoantibodies (17%)
Renal	28%	Autoimmune hepatitis (20%), glomerulonephritis, interstitial nephritis, unexplained hypertension, persistent hematuria, proteinuria,
Neurologic	24%	seizure disorders (14%), ventriculomegaly (3%), developmental delay (14%; higher than in other cohorts)
Pulmonary	23%	Asthma & interstitial lung disease (23%), lymphadenopathy (14%), lymphadenopathy (3%)
Hepatic	20%	
Arthritis/Vasculitis	9%	
Lymphadenopathy	14%	
Cardiovascular	2%	pericarditis, atrial flutter, dilated aortic root, pericardial effusion, and aneurisms
Failure to thrive	75%	

4 TREG CELL EFFECTOR FUNCTION

Treg cells are important for maintaining immune homeostasis, tolerance and control normal immune responses, due to their capacity to suppress a large number of immune cells, including T cells, DCs and NK cells (Levings et al., 2001). However, this ability also makes their malfunction susceptible to contribute to the development of autoimmune disorders. Furthermore, Treg cells are often associated with worse cancer prognosis (see section 5). In recent years, different mechanisms were postulated regarding how Treg cells execute their suppressive role. A wide repertoire of suppressive mechanisms ranging from cell-cell contact depending to cytokine-mediated suppression were described (Figure 4) (Vignali et al., 2008; Walker and Sansom, 2011). One drawback in studying suppressive function of human Treg cells is that research is often restricted to *in vitro* assays. Furthermore, most *in vivo* data are based on animal models.

One key role of Treg-mediated suppression is their ability to control the T cell pool by either directly interfering with T cell function or in a more indirect way via APC suppression. As discussed above Treg cells follow a similar path for cell activation as other CD4⁺ T cells, including their need for IL-2. Since Treg cells do not produce IL-2 themselves, they rely on IL-2 produced in their environment. A mechanism referred to as **IL-2 deprivation** was postulated by which Treg cells starve IL-2 dependent cells, including Teff cells, from IL-2 due to their continuously high expression of CD25 (Thornton and Shevach, 1998). However, IL-2 deprivation was shown *in vitro* and data that prove this concept *in vivo* are limited and complete IL-2 knockout mice die from a CD4⁺ T cell driven lymphoproliferative disease. Thus, it is not obvious to envision how IL-2 deprivation would function as a specific pathway to immunosuppression *in vivo* (Pandiyani et al., 2007).

Another mechanism of Treg-mediated-suppression of Teff cell activity is by co-expressing the extracellular nucleotidases (ectoenzymes) **CD39 and CD73**. CD39/CD73 converts ATP (adenosine triphosphate) to adenosine and thereby increasing pericellular adenosine which in turn generates an immunosuppressive environment for Teff cells due to their expression of the adenosine A2A receptor (Deaglio et al., 2007; Kobie et al., 2006; Dwyer et al., 2007; Zarek et al., 2008). Lately, the use of blocking antibodies against CD39/CD73 came into focus as a way to improve anti-cancer responses (Perrot et al., 2019).

Treg cells are also known to secrete anti-inflammatory cytokines, like IL-35, IL-10 and TGF- β . **IL-10** is a known anti-inflammatory cytokine, that beside T cells is produced also by B cells and monocytes. The actual contribution of IL-10 to Treg suppressive functions is still unclear (Asseman et al., 1999; Hara et al., 2001; Rubtsov et al., 2008). Beside the role of IL-10 in Treg development, **TGF- β** production is also described as an effector function of Treg cells but similar to IL-10 its contribution and functional mechanism are unclear. However, *in vitro* assays the physical separation of Treg cells and responder cells results in reduced suppression and neither IL-10 or TGF- β knock out mice develop an autoimmune phenotype. Nevertheless, in Treg cells from IL-35 knock out models display a reduced functionality. And IL-35 alone shows the capacity to suppress T cell proliferation *in vitro*.

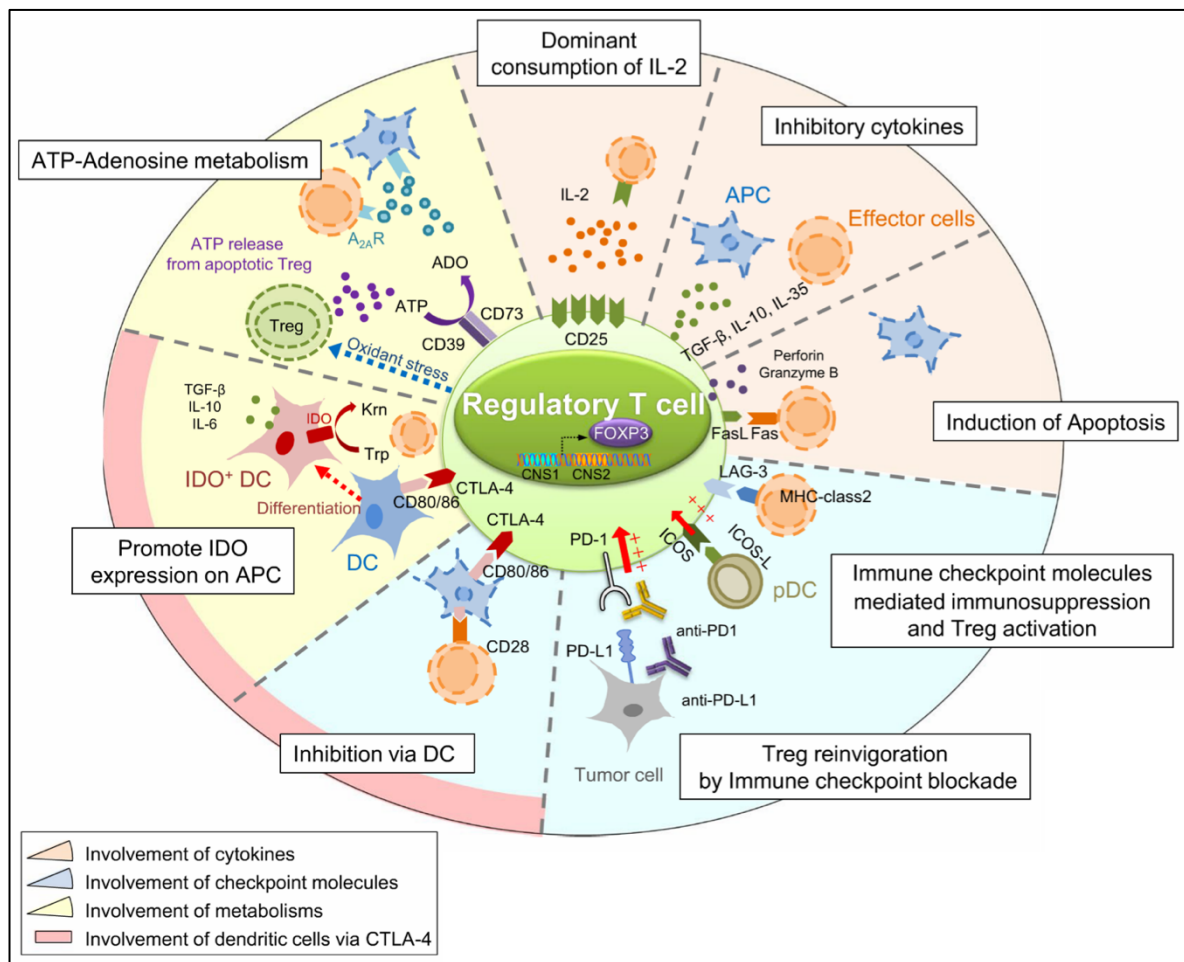


Figure 4: Treg cell mediated suppressor function. Treg cells exhibit a variation of effector functions ranging from secretion of anti-inflammatory cytokines, expression of checkpoint inhibition molecules and metabolic disruption. Target cells for Treg mediated suppression includes Teff cells and DCs. (Ohue and Nishikawa, 2019)

Another well studied mechanism how Treg cells can control Teff cells is by controlling Tn cell activation via the **suppression of DCs**. It is known from *in vitro* assays that DCs show decreased levels of CD80 and CD86 expression in the presence of Treg cells and these suppressed DCs are less capable to support T cell proliferation (Cederbom et al., 2000; Tang et al., 2006). Furthermore, it was shown that Treg cells interact with DCs *in vivo*. In the case of Treg-mediated suppression of DCs several mechanisms are described, including via the expression of CTLA-4, Lag-3 and Nrp-1 and induction of IDO in DCs. The enzyme Indoleamine 2,3-dioxygenase (IDO) turns tryptophan to kynurenine. The depletion of tryptophan from the environment inhibits Teff cell activation and proliferation. The induction of IDO expression in DCs was shown to be associated with CTLA-4 engagement with CD80 and CD86.

Beside CTLA-4 Treg cells express additional **immune checkpoint molecules** like programmed cell death protein 1 (PD-1), ICOS and Lag-3. PD-1 molecule is mainly expressed on B cells and T cells, including Treg cells, and belongs to the same superfamily as CD28, ICOS and CTLA-4 (Chamoto et al., 2017; Yamazaki et al., 2002). PD1 ligands include programmed cell death ligand 1 and 2 (PD-L1 and PD-L2) and are both expressed by a variety of immune cells, including DCs. Additionally PD-L1 can be also found on non-hematopoietic cells (Chamoto et al., 2017; Yamazaki et al., 2002a). More recent studies also indicated that PD-1 might interact with CD28 on CTLs. PD-1 ligand binding results in the blocking of the signaling cascade

downstream of TCR by e.g. intervening with PI3K signaling. Furthermore, PD-1 expression on T cells is also associated with cell exhaustion (Chamoto et al., 2017; Liang et al., 2006; Okazaki et al., 2011).

4.1 CTLA-4 (CD152)

In humans, the gene encoding CTLA-4 is located on chromosome 2q33 downstream of CD28, whereas in mice it is located on chromosome 1 (Brunet et al., 1987; Naluai et al., 2000). CTLA-4 is a structural homologue to CD28 that encodes both a binding domain for the co-stimulatory molecules CD80 and CD86 expressed on APCs and CTLA-4 binds with a ~20 fold higher affinity to CD80 than CD28. Both molecules belong to the same immunoglobulin family but CD28 functions as a co-stimulatory receptor whereas CTLA-4 is a negative modulator in T cell activation (Brunet et al., 1987; Freeman et al., 1991; Harper et al., 1991; Linsley et al., 1991b; 1991a; van der Merwe et al., 1997; Rudd et al., 2009; Tivol et al., 1995; Walunas et al., 1994; Waterhouse et al., 1995). CTLA-4 is a homodimer mainly expressed on CD4+ and CD8+ T cells. But several studies indicated that CTLA-4 can be expressed by APCs including B cells and potentially DCs. The functional role and general expression pattern in these cells still needs to be studied in greater detail (Brunet et al., 1987; Oaks et al., 2000; Quandt et al., 2007; Wang et al., 2011). In contrast, CTLA-4 expression in T cells and in particular Treg cells, is a well-established feature and is associated with Treg cell suppressive function. CTLA-4 is constitutively expressed on Treg cells in a FoxP3-dependent manner and its expression is rapidly further upregulated upon cell activation. Similarly, T_H1 cells start to upregulate CTLA-4 expression upon TCR stimulation but it is not expressed in naïve T cells (Miyara et al., 2009; Rudd et al., 2009; Takahashi et al., 2000). In T cells, CTLA-4 accumulates, if expressed extracellular, in the immune synapse but the majority is stored intracellular (in endosomes) and therefore can be rapidly upregulated. CTLA-4 can be re-cycled in cells and after internalization via endocytosis it is either stored and re-expressed or undergoes degradation in lysosomes. In recent studies it was shown that the protein LRBA (lipopolysaccharide-responsive and beige-like anchor protein) is heavily involved in recycling of CTLA-4 (Hou et al., 2017). Just as CTLA-4, CD28 also undergoes endocytosis and binding of PI-3K to the cytoplasmic tail of CD28 is involved in this process. Similarly, CD28 either re-cycles back to the cell surface or undergoes degradation (Céfaï et al., 1998).

The CTLA-4 protein consists of four functional domains encoded by four exons. The extracellular part consists of a leader peptide (encoded by exon 1) and a ligand-binding domain (exon 2), followed by a transmembrane domain (exon 3) and finally, the cytoplasmic tail (exon 4) that is part of the intracellular domain (Brunet et al., 1987; Ling et al., 1999). In mice, four **isoforms** were identified that are results of alternative splicing: a full length form of CTLA-4 (fCTLA-4), a ligand independent form lacking exon 2 (liCTLA-4), a soluble isoform lacking exon 3 (sCTLA-4) and a isoform lacking both exon 2 and 3 (1/4CTLA-4) (Figure 5). In humans, the liCTLA-4 isoform is not described (Huurman et al., 2007; Oaks et al., 2000; Ueda et al., 2003).

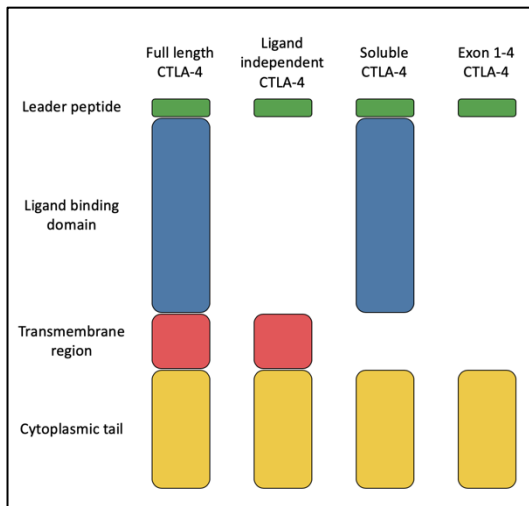


Figure 5: CTLA-4 isoforms. CTLA is encoded by four exons that each in itself encodes one functional domain. All exons can be alternatively spliced what gives rise to four different isoforms.

In a study done by Oaks et al. (2000) it was shown that **sCTLA-4** is conserved between mouse, rat and humans. mRNA expression levels for sCTLA-4 vary between immune cells (CD4+ T cells expressing the highest levels), cell activation (resting cells express pre-dominant sCTLA-4) and location (present in blood serum but not in thymus) (Oaks et al., 2000) and over-expression of sCTLA-4 was linked to several autoimmune disorders (Pesce et al., 2014; Saverino et al., 2010; Simone et al., 2014). In respect to **1/4CTLA-4** little is known about its function in a normal immune setting but in several studies, it was shown that 1/4CTLA-4 is over expressed in autoimmune patients (Ichinose et al., 2013; Liu et

al., 2012). The **liCTLA-4** isoform is the only one not described in human. In a diabetes mouse model it was shown that liCTLA is a naturally occurring isoform in peripheral T cells and has an enhanced expression on memory cells. Furthermore, this study suggests that liCTLA-4 plays a role in preventing T cell mediated autoimmunity. Furthermore, in a study done on mice charring an artificial mutation in the exon 2 region of CTLA-4 showed a loss of the binding capacity does not result in a lymphoproliferative phenotype in these mice. Additionally, they saw that CTLA-4 mediated suppression of T cell activity was not impaired (Chikuma et al., 2005; Stumpf et al., 2013; Vijayakrishnan et al., 2004).

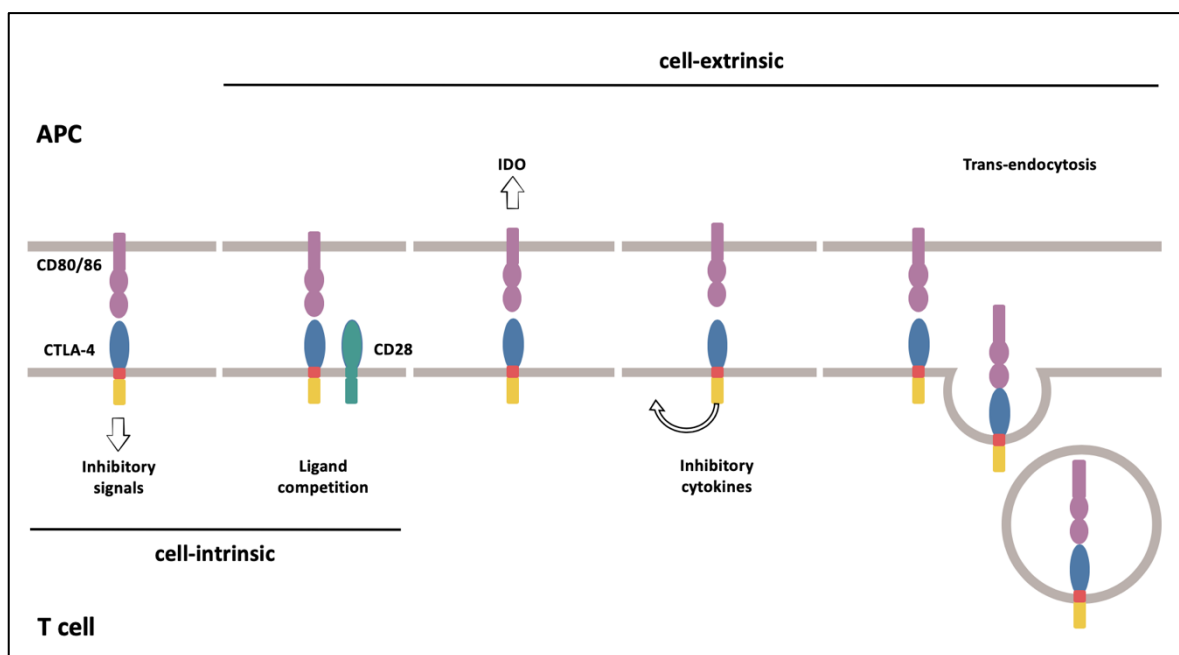


Figure 6: CTLA-4 signaling. Several mechanism of function were described for CTLA-4 ranging from cell intrinsic pathways to cell-extrinsic pathways.

The importance of CTLA-4 to control immune tolerance and maintain immune homeostasis is well established. In independent studies done on knock-out mice models it was shown that **CTLA-4 deficiency** leads to the development of an autoimmune phenotype similar to the scurfy phenotype. For example, germline CTLA-4 deficient mice died within 3 to 4 weeks after birth caused by lymphoproliferative disorders. (Chambers et al., 1997; Tai et al., 2007; Tivol et al., 1995; Waterhouse et al., 1995). Furthermore, *Wing et al.* (2008) studied the effect of CTLA-4 deletion in CD4⁺FOXP3⁺ Treg cells by creating a Cre-conditional CTLA-4 knock out strain under the FOXP3 promotor. When compared to complete CTLA-4^{-/-} mice, the conditional knock-out mice still develop a lymphoproliferative diseases phenotype but at a slower progression rate. Furthermore, Treg cells lacking CTLA-4 showed an impaired function in controlling CD80 and CD86 expression of DCs *in vitro* (Wing et al., 2008). In contrast, in conditionally ablated CTLA-4 during adulthood in CD4⁺ T cells, mice did not develop a fatal phenotype as seen in the germline deletion models. Nevertheless, histological data revealed pathological inflammatory changes in several tissues including insulinitis and gastritis (Klocke et al., 2016; Paterson et al., 2015). A comparable situation was described in humans where **polymorphisms** in the CTLA-4 gene were correlated with different autoimmune and inflammatory diseases.

Taken together these findings indicate that CTLA-4 is a major player in maintaining immune hemostasis and tolerance by controlling T cell proliferation. But until today it is still controversial how CTLA-4 functions and signals. Several models for CTLA-4-mediated suppression were postulated that can be divided into cell-intrinsic pathways and cell-extrinsic pathways (Figure 6). When it comes to CTLA-4 mediated suppression Treg cells due to their constitutively high expression, however since effector T cells express CTLA-4 as well they can contribute to CTLA-4 mediated suppression.

Several mechanisms were postulated in respect to **cell-intrinsic** suppression of CTLA-4 that could directly affect Treg cell function. It was shown that the **cytoplasmic tail** of CTLA-4 is not only involved in recycling of the protein but also in receptor signaling. Protein kinase C was shown to interact with the cytoplasmic tail, an interaction that supports Treg cell suppressive activity (Choi et al., 2006; Kong et al., 2014; Stumpf et al., 2014). Since CTLA-4 and CD28 are simultaneously expressed in the same cell, CTLA-4 could potentially outcompete CD28 signaling on the same cell.

Cell-extrinsic pathways focus on CTLA-4-dependent suppression of APCs. This includes IDO induction, secretion of inhibitory cytokines and controlling availability of CD80/CD86, all mechanisms are potentially Treg-mediated. In recent years, Treg cell-mediated inhibition of DCs gained more interest as it was discovered that Treg cells and DCs interact *in vivo*. DCs are known to promote T cell priming and differentiation, thus DC suppression could be one mechanism of Treg cells to indirectly control effector T cells *in vivo* (Tang et al., 2006). Moreover, it was shown that Treg cells can control CD80 and CD86 expression on DCs in both human and mice but these are mainly *in vitro* observations (Cederbom et al., 2000; Mavin et al., 2017; Misra et al., 2004; Onishi et al., 2008). Until now it stays unclear whether Treg cells actively downregulate CD80/CD86 or rather interfere with DC maturation. Several

mechanisms were proposed on how CTLA-4 controls CD80/CD86 availability during naïve T cell priming. Since CTLA-4 binds with a stronger affinity than CD28 and is highly expressed on Treg cells it was theorized that CTLA-4 simply outcompetes CD28 and thereby reducing co-stimulatory signaling. Another model that gained great popularity among researchers supports the hypothesis that the engagement of the ligand-binding domain of CTLA-4 with CD80/CD86 results in the physical removal of the receptors from the DC membrane in a process called transendocytosis (Hou et al., 2015; Qureshi et al., 2011; Walker and Sansom, 2011). *Qureshi et al.* (2011) introduced the concept of **transendocytosis** in the setting of CTLA-4 function. In an *in vitro* assay they demonstrated that CTLA-4-expressing CHO cells were capable of removing and internalize CD86 from CD86-expressing CHO cells. They furthermore demonstrated in a co-culture assay of MDDCs with activated human CD4+CD25- cells that CD86 was reduced on MDDCs and was found intracellular of activate T cells, a process that could be blocked by the use of anti CTLA-4 blocking antibody (Qureshi et al., 2011).

Regardless of cell-intrinsic or extrinsic pathways, what most of these mechanisms have in common is, that the engagement of the ligand-binding domain with CD80/CD86 is the cause of the signaling. The two isoforms 1/4CTLA-4 and liCTLA-4 lack the ligand-binding domain and until today their role is mainly discussed in pathological situations which warrants further study.

5 TREG CELLS IN DISEASE

Immunity regulates and contributes to most of today's major diseases. Thus, it is not surprising that a cell population, like Treg cells, that controls the magnitude of immune responses alter disease outcome in a wide variety of diseases. In autoimmune disorders Treg cells are seen to have a decreased capacity to suppress due to impaired function or numbers (Dominguez-Villar and Hafler, 2018). This reflects a setting where functional Treg cells are beneficial for the patient. On the other hand, in tumor biology the presence of Treg cells often predicts a poor prognosis. Even if this "either-or" picture reflects a simplified version of Treg cells function it still gives us an idea how Treg cell biology affects such a broad range of disease processes.

Many studies showed **cancer** is an umbrella term for a set of diseases that have the formation of tumor cells in common but are diverse in their cause, progression and treatment. Six hallmarks of cancer biology were originally postulated that characterize cancer cells: sustaining proliferation, evasion of growth suppression, invasion and metastasis, replicative immortality, induction of angiogenesis and resistance to cell death. Additionally, based on studies from the last two decades additional hallmarks (deregulation of cellular energetics and avoiding immune destruction) and enabling characteristics were added (tumor-promoting inflammation and genomic instability and mutations) (Hanahan and Weinberg, 2011). In respect to the immune system, it was shown that an inflammatory environment contributes to the development and progression of cancer and that tumor cells can develop a large repertoire of mechanisms to manipulate immune responses in their favor (Grivennikov et al., 2010). This includes the recruitment of suppressor cells like Treg cells and myeloid-derived suppressor cells (Gabrilovich and Nagaraj, 2009; Liu et al., 2016; Ohue and Nishikawa, 2019).

A large number of experimental studies have demonstrated that Treg cells inhibit anti-tumor immune responses (Onizuka et al., 1999; Teng et al., 2010). deLeeuw and colleagues recently reviewed 58 clinical studies and determined that Treg cells correlated with a negative prognosis in 23 studies, with a neutral prognosis in 23 studies and good prognosis in 12 studies (deLeeuw et al., 2012). Hence it seems that Treg cells play a two-faced role in anti-tumor responses. This might be explained by several things. It can be difficult to truly distinguish Treg cells from Teff since Teff cells are seen to transiently express FOXP3 in such highly activated environments and Treg cells are described to be a heterogeneous population, hence their functional state and stability might be more important than looking at total Treg cell number. Treg cells can also be suppressive towards other anti-tumor immune cells and thereby contribute to an immune suppressive tumor micro-environment (Whiteside, 2008). However, Treg cells can also suppress tumor promoting factors such as chronic inflammation as well as tumor invasiveness (Winerdal et al., 2018).

In the last couple of years **immune check point inhibition** showed to be a novel approach in treating a variety of cancer types (Anderson and Rapoport, 2018; Byun et al., 2017; Chamoto et al., 2017; de Coaña et al., 2015). Blocking antibodies against both PD-1 (Nivolumab) and

CTLA-4 (Ipilimumab) were approved for clinical use to target Treg cell function. Ipilimumab immune therapy showed promising results in some tumor settings but failed in others. In metastatic melanoma patients Ipilimumab alone was shown to lead to an enhanced overall survival. However, especially the combined usage of both anti-PD-1 and anti-CTLA-4 is promising (Curran et al., 2010). Nevertheless, immune check point inhibition comes with the risk of severe site effects in e.g. the liver, skin, and gut. This indicates how important it is to gain a better understanding in CTLA-4 signaling.

As seen in IPEX patients, the loss of Treg cells results in the development of a range of **autoimmune diseases** due to break of immune tolerance. This in turn results in an inappropriate reaction of the immune system to self-antigens and the development of chronic inflammation. Autoimmune diseases range from tissue specific disorders like type 1 diabetes and multiple sclerosis, systemic disorders like systemic lupus erythematosus (Miyara et al., 2011). In respect to Treg cells, defects in either cell frequency or function was linked to autoimmunity in human patients. (Dominguez-Villar and Hafler, 2018). Of special note for this thesis is atherosclerosis, which is a chronic inflammatory disease characterized by the formation of arterial plaques. A number of different immune cells infiltrate these plaques including macrophages, DCs and T cells. Over time the plaque hardens and narrows the arteries limiting the flow blood. Even worse, these plaques can rupture resulting in thrombus formation and vessel occlusion as seen in myocardial infarctions and stroke. CD4⁺ T cells promote plaque formation and decrease stability of atherosclerotic lesions, which consequently affects the clinical outcome of this disease. It is becoming increasingly clear that Treg cells inhibit plaque growth in murine models of atherosclerosis. Interestingly, an increasing amount of clinical studies do indeed suggest an association between higher numbers of Treg cells and a more favorable disease prognosis. Substantial efforts are currently being made to utilize Treg-based therapeutic approaches to cure inflammatory diseases including atherosclerosis (Ait-Oufella et al., 2006; Andersson et al., 2010).

6 STUDY AIM

The overall aim of this thesis is to gain a better understanding on how Treg cells suppress immune responses and how FOXP3 isoforms regulate immunity.

In **paper I** we studied the role of Treg cell mediated suppression of dendritic cells and in particular the role of the inhibitory receptor CTLA-4.

Paper II to IV focus on the function of FOXP3 isoforms. In **paper II** we studied the role of FOXP3^{fl} and FOXP3 Δ 2 in atherosclerosis. Furthermore, in **paper III and IV** we studied the FOXP3 Δ 2 isoform in two related IPEX patients to get a better understanding how FOXP3 Δ 2 acts in vivo. In paper III we studied the clinical manifestation and phenotype of Treg cells and in paper IV we focused on transcriptomic analysis of FOXP Δ 2 expressing Treg cells.

7 MATERIAL AND METHODS

The majority of the techniques used in this thesis are commonly used tools within the immunological and medical research fields. Unique methods include the generation of our *Ctla4^{ex2fl/fl}Foxp3-Cre* mouse strain, the in vitro DCs suppression assay in paper 1 and methods for FOXP3 isoform detection used throughout paper II – IV. For a more detailed description of these techniques see sections below.

Table 2: summarized the methods used per paper and for a more detailed description see corresponding papers.

Table 2: Methods used in study 1 to 4.

Methods	Paper			
	I	II	III	IV
Bone marrow chimeras	x			
Chromatin immunoprecipitation		x		
Clinical patient data		x	x	
Cytokine Bead array	x			
Flow cytometry-based cell sorting	x	x		x
Human specimens		x	x	x
In vitro cell assays	x	x		
Lentiviral transduction		x		
Mouse models	x			
Multi-color flow cytometry	x	x	x	x
PCR	x			
qPCR		x		
RNA sequencing	x	x		x
Sanger sequencing			x	
SDS-PAGE and western blot		x		
X-inactivation methods			x	

7.1 GENERATION OF CTLA4^{EX2FL/FL}FOXP3-CRE MOUSE

We generated conditional *Ctla4*^{ex2fl/fl}Foxp3-Cre knockout mice by inserting LoxP sites on both sides of exon 2 of CTLA-4. In brief, a targeting construct was generated from a C57BL/6 bacterial artificial chromosome (BAC) (clone RP23:388N14). The construct had a long homology arm which extended 6.01 kb 5' to the single LoxP site and the short homology arm extended 2.02 kb 3' to the LoxP/ FRT-flanked neo cassette. The targeting construct was linearized using NotI prior to electroporation into C57BL/6 embryonic stem cells, the stem cells were then microinjected into blastocysts from BALB/c mice. To remove the Neo cassette, the chimera was crossed with C57BL/6 Flp mice. *Ctla4*^{ex2fl/fl} mice were mated with Foxp3-Cre mice (C57BL/6/J background) to generate *Ctla4*^{ex2fl/fl}Foxp3-Cre mice (Figure 7).

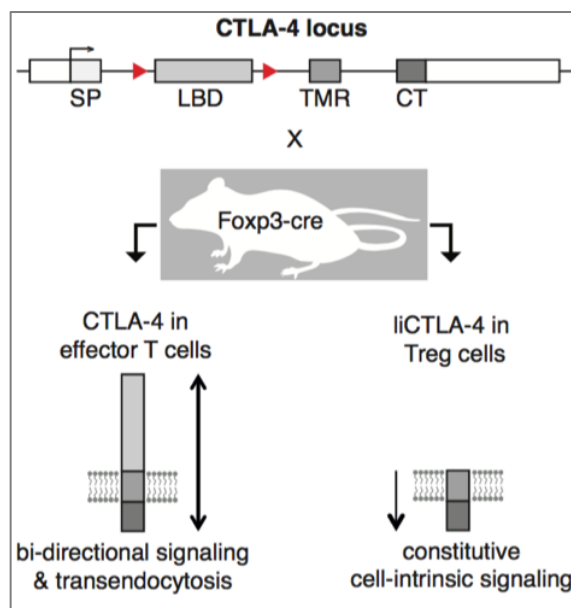


Figure 7: The *Ctla4*^{ex2fl/fl}Foxp3-Cre mouse strain is homozygote for Cre. Breeding pairs *Ctla4*^{ex2fl/fl}wtFoxp3-Cre to generate wild type litter mates and *Ctla4*^{ex2fl/fl}Foxp3-Cre animals.

7.2 IN VITRO DCS SUPPRESSION AND T CELL PROLIFERATION

For **CD4⁺CD25⁺ Treg cells** a single cell suspension was prepared from peripheral LNs (superficial cervical, axillary, brachial and inguinal) as well as mesenteric LNs. Cells were enriched for CD25 using CD25-PE antibody and anti-PE MicroBeads (Miltenyi Biotec). For the enrichment step either manual selection columns or the AutoMACS positive selection program was used. CD25⁺ enriched cells were then labeled with CD4-APC antibody and CD4⁺CD25⁺ Treg cells were sorted using flow cytometric cell sorting. For **Treg cell activation** cells were pre-activated for 3 days. In brief, cells were cultured for 48h in the presence of 5 µg/mL plate-bound anti-mouse CD3, 500 U/mL recombinant IL-2 and rested for 24h in 500 U/mL recombinant IL-2. **CD4⁺CD25⁻ Effector T cells** were isolated in a similar manner to Treg cells from single cell suspensions prepared from LNs.

For **CD11c⁺ DCs** isolation a single cell suspension was prepared from spleens. T cells, B cells and NK cell were depleted by labeling cells with a cocktail antibody mix in combination with anti-biotin beads and using depletion columns (Miltenyi). The negative fraction was labeled with anti-CD11c⁺ micro beads (Miltenyi Biotec) and CD11c⁺ DCs were positively selected using a manual column (Miltenyi Biotec).

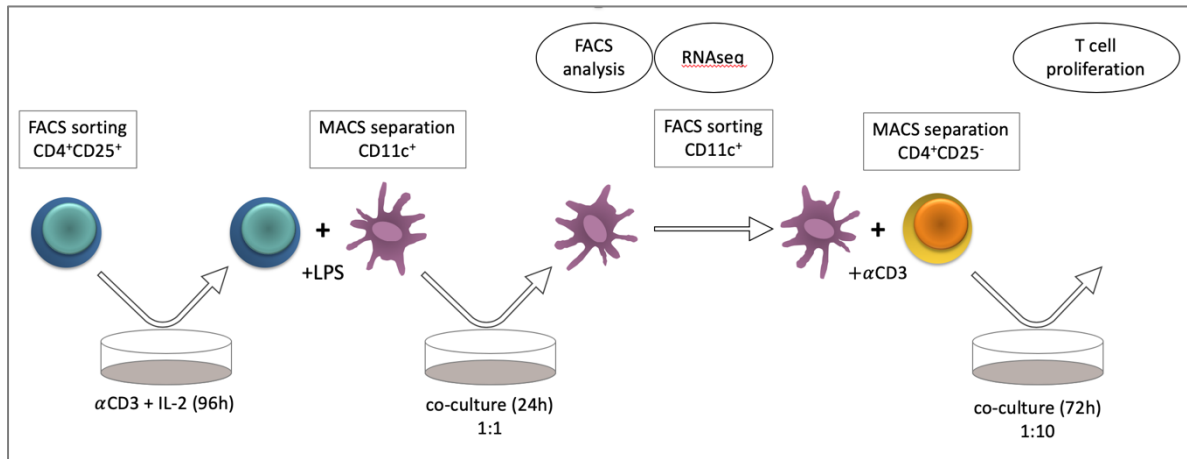


Figure 8: *in vitro* DCs suppression assay and T cell proliferation assay

Pre-activated Treg cells were **co-cultured** in a 1:1 ratio with CD11c⁺ DCs in the presence of 100 ng/ml LPS. After 24 hours cells were either analyzed using flow cytometry or resorted for either T cell proliferation assay or RNAseq. For the T cell proliferation assay DC were co-cultured with effector T cells in a 1:10 ratio (DC : Teff) in the presence of 0.25μg/ml soluble αCD3. After 72 h cell proliferation was measured by thymidine incorporation (Figure 8).

7.3 FOXP3 ISOFORM DETECTION

In this thesis, three methods were used to detect FOXP3 isoforms, namely qPCR, flow-cytometry and western blot. All three methods have technical limitations but combined give a reasonable quantification of isoform expression (Figure 9).

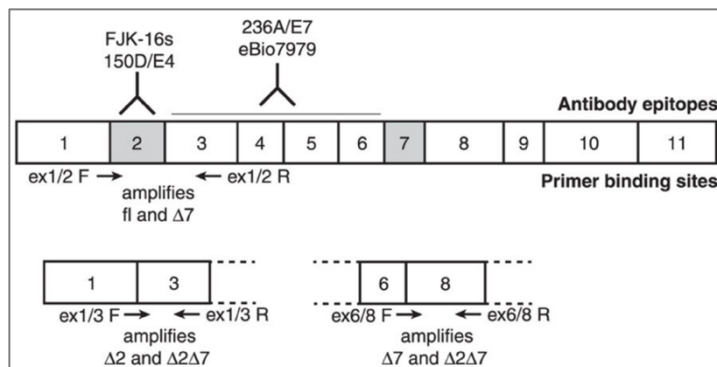


Figure 9: Detection of FOXP3 isoforms using antibodies and PCR primer pairs.

A combination of different primer pairs (Table 3) that bind to exon boundaries were used to calculate FOXP3 expression using qPCR. As primer pairs are not equally efficient in amplifying DNA we determined and corrected for the efficiency for the different pairs. The sum of FOXP3ex1-2 and FOXP3ex1-3 (FOXP3Δ2) was used to calculate total FOXP3 mRNA expression. Splice-variant ratio was calculated as followed: (FOXP3variant/FOXP3total)*100.

Table 3: FOXP3 primers for exon boundary detection.

Exon detection		Primer sequence
exon 2+	forward	5'--- TTC ATG CAC CAG CTC TCA AC---3'
	reverse	5'---GCC TTG AGG GAG AAG ACC---3'
exon 7-	forward	5'---GAG CAG CAG GCA TCA TCC G---3'
	reverse	5'---CTG GGA ATG TGC TGT TTC C---3'
exon 2-	forward	5'--- GGA GGG CTG CAC CCA AAG ---3'
	reverse	5'--- CCG TTG AGA GCT GCA GCT GC ---3'

On protein level we detected FOXP3 using the antibodies summarized in Table 4. For flow cytometry two antibody clones were used to either detect total FOXP3 expression using 236A/E7 (FOXP3_{tot}) or the presence of exon 2 using the 150D/E4 (FOXP3_{ex2}) clone. The latter one was used to detect FOXP3_{fl}. MFI values of both FOXP3_{tot} FOXP3_{ex2} were first normalized to naïve T cells. FOXP3_{tot}/FOXP3_{ex2} ratio was calculated using normalized MFI values. Alternative splicing of exon 7 can be best studied at transcriptomic level since no currently commercially available antibody exists that recognizes exon 7.

Table 4: antibody clones for FOXP3 detection

Clone	Epitope side	Application	Species
150D/E4	exon 2	FACS	Human
236A/E7	Region exon 3 - 6	FACS	
eBIO7979	Region exon 3 - 6	Western Plot	
FJK-16s	exon 2	FACS	mouse

In western blot we used an antibody clone detecting all FOXP3 isoforms. FOXP3 isoforms were distinguished based on molecular weight with FOXP3_{fl} having the biggest one (47 kDa). FOXP3 Δ 7 (44 kDa) and FOXP3 Δ 2 (43 kDa) are too close in their molecular weight to be properly distinguished. Both FOXP3 Δ 7 and FOXP3 Δ 2 Δ 7 (40 kDa) are both expressed at a too low level (based on data from qPCR) to be reliably quantified using this method.

8 RESULTS AND DISCUSSION

8.1 PAPER I

It is well established that Treg cells constitutively express the coinhibitory molecule CTLA-4, which is essential for Treg cells to function. However, it remains controversial as to how CTLA-4 exerts its function. In this study we generated a conditional knockout mouse model to study the relative contribution of cell-intrinsic and cell-extrinsic actions mediated by CTLA-4 on Treg cells. These ***Ctla4^{ex2fl/fl}Foxp3-Cre* mice express only the naturally occurring ligand independent isoform of CTLA-4 in Treg cells.** This CTLA-4 isoform is unable to interact with CD80 and CD86 on antigen presenting cells. Importantly, the expression of CTLA-4 was not affected in other immune cells as seen by the normal CTLA-4 expression in effector T cells. As previously shown, CTLA-4 is an important marker for Treg function and mice lacking complete CTLA-4 expression either specifically in Treg or as germ line deficiency, die from lymphoproliferative disorders. One of the key findings in this study is that ***Ctla4^{ex2fl/fl}Foxp3-Cre* mice did not develop a lymphoproliferative phenotype.** Instead, they appeared overall healthy at 6-10 weeks of age similar to their wild type litter mates. In contrast, mice completely devoid of CTLA-4 in Treg cells developed lymphoproliferative disease. The previous study by *Wing et al.* demonstrated that mice completely devoid of CTLA-4 in Treg cells die at 10 weeks of age due to lymphoproliferative disease. We believe the discrepancy seen between these studies is that our mice were on a C57BL/6 background whereas the previous study used a BALB/c background. When we extended our study, we saw that **older *Ctla4^{ex2fl/fl}Foxp3-Cre* mice (6 months) developed an inflammatory phenotype in the lung.** It is tempting to speculate that this is due to a different functional phenotype of peripherally induced Treg cells as they are believed to play a role in maintaining tolerance at environmental exposed surfaces including the lung. To answer this question, we could transfer CD45.2+CD4+GFP- cells from a *Ctla4^{ex2fl/fl}Foxp3-CreFoxp3-GFP* reporter mouse to a CD45.1+ host and study function. Furthermore, we saw an increased thymic output of Treg cells. This is in contradiction to studies done in mice having LoxP3 sites around exon 2 and 3 of CTLA-4 (*Wing et al., 2008*). This may be due to differential efficiency of the Cre-mediated recombination between the different studies, but it remains unknown why that would be the case. Furthermore, it was also shown that when CTLA-4 deletion is introduced during adulthood, mice do not develop a lethal phenotype (*Klocke et al., 2016; Paterson et al., 2015*)

We did not see an increase in Treg cell numbers nor any change in the expression of co-stimulatory molecules on DCs. However, ***Ctla4^{ex2fl/fl}Foxp3-Cre* mice had an increased number of Treg cells, in particular with Treg cells lacking CD25 expression.** The dramatic increase of Treg cells could potentially compensate for any functional impairment due to altered CTLA-4 expression. Thus, we isolated Treg cells for *in vitro* assays to further investigate Treg cell function and using an DC suppression assay that we established. We found that **Treg cells isolated from *Ctla4^{ex2fl/fl}Foxp3-Cre* were able to control CD80 and CD86 expression.** When it comes to CTLA-4 mediated suppression, transendocytosis has suggested to be an important functional mechanism. Our data does not contradict that transendocytosis can happen but it

supports the idea that it is not an essential pathway for Treg cell mediated suppression. Here it is important to note that most of the studies concerning transendocytosis have not used Treg cells but rather Teff cells or transduced cell lines. Also, we have been unable to replicate some of these findings. I strongly believe that these experiments should be repeated in a context with actual Treg cells before we speculate that transendocytosis contributes to the suppressive ability of Treg cells. Interestingly, **Treg cells isolated from *Ctla4^{ex2fl/fl}Foxp3-Cre* failed to support upregulation of PD-L2 on suppressed DCs.** We speculate that this is due to the interaction of CTLA-4 with CD80 and/or CD86. A potential next set of experiments would be to address this question by isolating DCs from CD80/CD86 knock out mice and use them in our in vitro suppression assay.

When we looked at Treg cell mediated suppression of DCs to determine whether Treg cells either suppress the maturation of DCs or DCs function we found that **Treg cells do not inhibit the maturation process but rather induce an anti-inflammatory program in these cells.** For this part of the study we used RNAseq data from DC suppressed by wild type Treg cells. We originally were planning to include DCs suppressed by Treg cell isolated from *Ctla4^{ex2fl/fl}Foxp3-Cre* mice, however the RNA-sequencing from this group unfortunately failed. Changes in RNA transcription does not automatically indicate corresponding protein levels will be altered (Vogel et al., 2010). Therefore, it would be interesting to see how Treg cells might affect the induction of an anti-inflammatory phenotype on additional levels of the gene expression pathway, for example by regulation of mRNA translation. Furthermore, it was shown that LPS exposure in DCs results in epigenetic changes (Saeed et al., 2014). Ideally it would be interesting to also focus on epigenetic changes.

Since Treg cell sorting for functional assays rely on extracellular markers we were not able to include FOXP3⁺CD25⁻ cells to assess their function. It would be possible to backcross our mice strain to mice expression GFP under the FOXP3 promoter (Fontenot et al., 2005b). This would give us the possibility to use GFP in combination with CD25 to isolate both FOXP3⁺CD25⁺ and FOXP3⁺CD25⁻ cell populations. However, due to limitation in both time and resources we did not pursue this line of experiments. In this regard it would be of general interest to establish the transcriptional profile of these *Ctla4^{ex2fl/fl}Foxp3-Cre* Treg cells to answer the question how they differ in respect to wild type cells.

When it comes to CTLA-4 research, most studies do not account for the presence of isoforms. This can be partly due to the limitation in methods. Antibodies available for flow-cytometry detect epitope in the ligand binding domain only, whereas there are antibodies clones described for western blot that bind to the cytoplasmic tail. However, when we attempted to use these antibodies to study any potential signaling of liCTLA-4 we were unable to get them to work. An alternative strategy to study CTLA-4 signaling would be to introduce a biochemical tag such as a FLAG tag. We know the consequences of failed CTLA-4 signaling but it is still not fully understood how CTLA-4 signals.

In summary, we do not rule out that transendocytosis may occur, but it remains to be seen whether it contributes to the suppressive ability of Treg cells. In fact, our data suggests that this may not be the case. One major part that is lacking in respect to CTLA-4 is a better

understanding of its signaling, especially in the context of liCTLA-4. Moreover, little is known about the functional role of both liCTLA-4 and 1/4CTLA-4. In contrast it was shown that sCTLA-4 seems to be more prevalent in naïve T cells and that fiCTLA-4 is upregulated during T cell activation. It would have been interesting to see if cell activation alters the ratio between expressed CTLA-4 isoforms.

8.2 PAPER II

When it comes to studying FOXP3 isoform we face several limitations with regards to available reagents. Techniques to detect FOXP3 mRNA species include qPCR, mRNA sequencing (both single cell and bulk) and Northern blot. On protein level methods like flow cytometry and western blot rely on antibody binding and are widely used. Commercially available clones only bind either exon 2 expressing isoforms or all four isoforms simultaneously. So far, our efforts to produce an antibody binding in the exon 7 region has not been successful. I believe that one of the most important advances in studying FOXP3 isoforms would be to generate monoclonal antibodies towards the exon/exon junctions in the various FOXP3 isoforms. Such antibodies could potentially be used for both western blot and flow cytometry and would greatly facilitate studies on expression of FOXP3 isoforms.

One of the key findings in this study is that **during Treg cell activation the overall upregulation of FOXP3 expression is mainly due to an increased expression of the isoforms FOXP3Δ2**. These findings were confirmed using qPCR and on protein level using Flow cytometry and Western blot. However, this contradicts the findings in the recently published study by Lundberg et al. (2017) where they showed that increased FOXP3 expression after TCR stimulation was mainly due to an increase in exon 2 expression (Lundberg et al., 2017). It is noteworthy that the latter study did not study FOXP3 expression in Treg cells, but rather Teff cells, nor did they confirm their results by either western blot or PCR. It is also unclear how the quantification of FOXP3 isoforms is performed as the reference lines displayed are clearly off the population centers in the FACS density plots.

It is difficult to assess the role of FOXP3 isoforms in an *in vivo* situation since mice do not express similar isoforms. However, in a study published in 2015 a mouse model with a knock-in construct replacing FOXP3^{fl} with a version lacking exon 2 and 7, FOXP3^{Δ2Δ7}. These mice developed a phenotype similar to scurfy mice and this finding supports *in vivo* data from human Treg cells that showed that the lack of exon 7 results in the loss of FOXP3 suppressive function (Joly et al., 2015). A similar approach could be used to study FOXP3Δ2. Such a mouse model would not only give us tools for *in vivo* experiments but also answer the question of whether the observed phenotype of mice is due to the forced expression of an isoform that does not exist naturally in mice or it is a result of functional differences between FOXP3 isoforms.

Another key finding in this study is that **increased FOXP3Δ2 expression correlates with better plaque stability** indicating that aTreg cells might limit disease progression. This demonstrates

the importance to not only use total FOXP3 expression as marker for Treg function and that Treg cell number alone does not necessary correlate to the suppressive activity. Interestingly, **we did not find this correlation when looking at peripheral blood Treg cells and plaque stability**. This illustrates the limitation of studying peripheral blood and the need to include additional specimens when possible.

One limitation of this study is we study gene expression of bulk cells. This created challenges to correlate the expression of molecules induced by FOXP3 Δ 2, such as GARP, with disease progression as they can be expressed by non-Treg cells. One way to address the question regarding heterogeneity of gene expression in plaque cells would be to run single cell RNA-seq of either all cells, T cells or Treg cells. Bulk sequencing of sorted T cells or Treg cells would also be helpful. As we are working with large patient cohorts this is currently cost-prohibitive. It may also be possible to estimate the Treg contribution to total gene expression by computational methods such as of complete deconvolution CDSseq (Monaco et al., 2019). All these strategies would allow us to specifically correlate gene expression in Treg cells with vascular symptoms.

In summary, we found that FOXP3 Δ 2 expression correlates with better atherosclerotic plaque stability and as expected FOXP3 Δ 2 controls a subset of genes partially different from FOXP3fl. A natural next step is to address the function of FOXP3 Δ 2 regulated genes in either animal models for atherosclerosis or clinical samples. I favor the latter strategy as the mice do not express Foxp3 isoforms and animal models for atherosclerosis do not exhibit unstable plaques.

8.3 PAPER III & IV

Studying human diseases give us the option to get a better understanding of biological processes *in vivo*. FOXP3 isoform research in human is mainly conducted using healthy controls that express both isoforms. Hence, studying single isoforms is either restricted to *in vitro* experiments or artificial models using mice. IPEX by itself is a rare disease and in study 3 and 4 we had the unique opportunity to study the effect of a novel mutation (**NM_014009.3:c.305delT**) in exon 2 that results in a frameshift and the loss of FOXP3fl and any isoform expressing exon 2. As a consequence, **Treg cells in this patient express only one functional suppressive isoform, namely FOXP Δ 2 (and undetectable levels of FOXP3 Δ 7)**. One of the most striking findings is that **both patients have a relative mild phenotype in respect to their IPEX diagnosis**. In particular the older male did not show any IPEX related symptoms until his 40ies. Both patients carry the same mutation in FOXP3 but we cannot rule out that additional mutations, that we did not check for, contribute to the more severe phenotype in the younger male. Furthermore, environmental factor most likely contributed to the difference in clinical manifestation.

These patients show that FOXP3 Δ 2 by itself is capable to induce and maintain Treg cell differentiation. Based on the milder phenotype we at least know that these Treg cells have

an impaired function but are functional enough to prevent systemic fatal break of immune tolerance. However, looking at the female carrier we saw that only around 20% of Treg pool where c.305delT Treg cells a ratio that cannot be explained by random X-chromosome inactivation. We speculated that that either during Treg cell differentiation wild type Treg cells are favored or that c.305delT Treg cells do not survive in the periphery. One way to answer the question is to look at pTreg cell and tTreg cell development in both the patient and the female carrier. So far, the only partly repayable method to distinguish pTreg and tTreg cells is to look at epigenetic factors regulating the *Foxp3* gene. It would be interesting to see in the mother how wild type Treg cell differ in this respect to c.305delT Treg cells. Furthermore, a study the kinetics of FOXP3 expression *in vitro* by induction of FOXP3 in the presence of TGF- β and IL-2 could give us an indication if c.305delT Treg cells have impaired stability.

We looked at the transcriptional profile of rTreg cells and aTreg cells of both male patients and found indications that **c.305delT Treg cells display reduced lineage stability**. We found significant changes in genes (ID3, Bcl6, eIF4E and MAP4K8) associated with lineage plasticity when comparing with healthy controls. Furthermore, using gene set enrichment analysis, we found indications that **c.305delT Treg cells might have an impaired function in respect to mRNA processing**. Importantly, we had only two biological replicates from the IPEX group we and therefore used technical replicates for RNA-seq. This is not optimal but not unexpected as IPEX is such a rare disease and that results from multiple mutations. As the phenotype is mild it is unlikely that similar patients even will receive an IPEX diagnosis as exemplified by the advanced age at which our patients were diagnosed. The findings from these patients at least gives us an indication how FOXP Δ 2 works in a human *in vivo* setting. In case we would get more blood samples we potentially could look at FOXP3 Δ 2 binding partners and targets by e.g. ChIPSeq. Furthermore, we could design an *in vitro* setting similar as descried in paper 2 and compare findings from transduced cells with patient data.

9 CONCLUDING REMARKS

With an increased understanding of Treg cell biology we now know that Treg cells comprise a heterogenous population of cells. How Treg cells are defined in humans have evolved since they were rediscovered in the mid 1990s. From being known as CD4⁺CD25⁺ T cells to being defined CD4⁺CD25^{high}CD127^{low} with FOXP3 as a key regulator. This has further evolved with the discovery that Teff cells transiently express FOXP3 and that rather the de-methylated status of the FOXP3 locus determines stable FOXP3 expression in Treg cells. Furthermore, FOXP3 isoform expression introduces another level of complexity into Treg cell characterization. In this thesis we showed the functional differences between the FOXP3 isoforms FOXP3fl and FOXPΔ2 in respect to Treg cells activation and lineage stability. Furthermore, we demonstrated that Treg cells are capable to maintain immune homeostasis independent of CTLA-4 ligand binding and that CTLA-4 mediated cell intrinsic signaling is capable to induce an anti-inflammatory phenotype on DCs. Our findings challenge idea of the central role of the CTLA-4 binding domain in CTLA-4 mediated suppression.

10 ACKNOWLEDGEMENTS

Hej and welcome to the most important part of a thesis. It is difficult to put in words how thankful I am for all the people that accompanied and helped me during the last years.

John, thank you for all your support, I could not image a better supervisor. Thank you for all your encouraging “everything will be fine” (when nothing seemed fine) and all the “that’s character building” (even if that didn’t help at all). But the most I want to thank you for creating an environment where we can laugh about bad jokes and even at the misery that comes with research. Because as long as you can laugh, everything will be fine. Also, the movie nights broadened my horizon of amazingly awful movies.

I want to thank my co-supervisors and mentor: **Andreas**, when I first came to KI, it was a great pleasure to work in your group. You encouraged me to start a PhD and introduced me to John; I’m still not sure if I should be thankful for this or not, for everything else, thank you for sure! **Lisa**, the leader of the strange group from MTC putting up WASP stickers all over our lab fridges. Thank you for all your scientific input and help especially during the last couple of months of my PhD when John dropped me off at MTC. You showed me that B cells might be a tiny bit more interesting than I initially thought. **Richard**; the one person I never met. I am still interested in doing the experiments we initially planned for ☺. I also want to thank **Anna** for being my mentor. I like your color-coded office and I always enjoyed talking to you.

Special thanks to all the (now former) members of the T-rex Group. I miss our general meetings and working with you all was great! **Anne-Laure**, I admire your passion for science and I learned so much from you. Not only the importance of details (be aware of the fluffy pullover) but also to be opinionated about food. **Sang**, thank you for taking me under your wing when I joined the group and teaching me so much scientifically, introducing me to muji pens and most importantly your life lesson on the importance of coffee in the morning. Hopefully we can watch Mulan together! **Philip**, my first and only student that survived me more than a day. Not sure if it makes you brave or crazy. It was great working with you and I hope to see you back in Sweden soon. I would also like to thank the three honorable members of our group: **Fang**, for introducing me to ALS research. **Solmaz** (my toothless-hoody-twin), for all the time you shared your food with me and hopefully we will also share an office at some point ☺. **Can**, for help with experiments. Best of luck with your studies!

Thanks to everyone at **Allergy and Immunology** unit that created such a great environment to work. **Jennine**, I learned more of bikes and racing from you than I ever wanted to know and thanks to you I now know how cool mast cells can be. **Casper**, you are an incredible patient person and I don’t know who else could stand me and bubble-wrap together in one room. **Avinash**, thanks for all the talks and the Indian tea. I still think our poking device is the best invention ever! **Katarina**, I’m happy you are back from Huddinge so we can have lunch more often together. When one day we face a Zombie apocalypse (thanks to you and John) I will hide with you! Until then thanks for your help with all this grown up stuff. **Maria**, all the talks we had over coffee always cheered me up! **Sindhu**, I enjoyed dropping by your desk in

the morning and all our conversations about Harry Potter and brainstorming over Marvel movies. Everyone from Eduardo's group, thanks for your help and input with the mice project and the Monday morning JCs as long as it lasted. **Martina**, hope you're not stressing too much, about your Thesis, you will do great! I also want to thank Susann's group for Mr Hippo, who is BTW not a pig. **Annika**, **thank you a lot** for all the help during these years!

Also special thanks to the WASP group that "adopted" me the last couple of months during my PhD. **Marianna, Ming, Julien, Nik, Larissa, Chiara, Christoph** and **Marton**, thanks for the warm welcome.

Working in research means working in teams and I had the pleasure to work with great people. Thanks to all the collaborators on the CTAN project. **Kajsa, Katrin** (yes, I love your thesis cover!), **Charlos, Kristina, Eduardo, Paulo, Martina, Jonathan** and **Chris**. Also, many thanks to everyone who worked with us on all the FOXP3 projects and are equally convinced isoforms are cool: **Katie, Paul, Cindy, Stuart, Zuzana, Charles, Göran** and **Karl**.

I want to thank all my friends that make Sweden a second home. **Solmaz, Filip and Nicki**, staying with you always feels like a mini vacation, thanks for all your support and the amazing food! Hopefully we will fix the fireplace soon so I can burn my thesis and that we continue with Pandemic while listening to sound of paper burning. **Anne-Laure and Nina**, I'm looking forward to have many more crafting evenings together, where the crafting is maybe more a cover to drinking wine and talk. Also, thanks backing me up that Chris should definitely cook more ☺. **Alex**, thank you for letting me play with your stuff, you are also the one person I know who loves dragons as much as I do. **Mathilda**, thanks for telling John he should be nice and your help with the thesis cover. **Kristina and Laia**, thanks you for your friendship and making Stockholm such a welcoming place when I first arrived in Sweden. Thank you for all the time we baked, cooked and watched ESC together! Kristina, also thanks for letting me stay with you when I (again!?!) lost my keys and hopefully one day we will hear "shut up and dance" live. **Nicole, Matthew, Frejya** (the fierce) and **Leo**: I really enjoyed our trips together and the shooting lessons. You made America great (again) ☺ **Linda, Tom, Naemi** and **Sirre**: thank you for the nice times climbing and the time spent together. I hope to do more outdoorsy stuff with you guys.

To my family: **Mama & Paps**, danke für all eure Unterstützung in allem was ich mir in den Kopf gesetzt habe. **Susanne**, best big sister since 30 years and still going strong! Hoffe wir sehen uns früher als geplant ☺ **Antoine** Thanks for all the great movie suggestion! **Louise**: je ne suis pas petite Tina! **Chris**, there are not many people I can imagine to have a 10 hours car drive with after being awake for a day followed by setting up a tent in the middle of the night, that I do not want to strangle. But you are one of them and I hope there will be many more 10 hours drives and travels (where I hopefully not strangle you, but only kick when you snore). Thank you for making sure that I had food (not snacks) and my daily dose of cute cat and dog pictures. But the most, thank you for being you and for giving me the comfort that I can be me around you.

11 REFERENCES

- Aaltonen, J., Björnses, P., Perheentupa, J., Horelli-Kuitunen, N., Palotie, A., Peltonen, L., Lee, Y., Francis, F., Henning, S., Thiel, C., et al. (1997). An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nat Genet* 17, ng1297-399.
- Ait-Oufella, H., Salomon, B.L., Potteaux, S., Robertson, A.-K.L., Gourdy, P., Zoll, J., Merval, R., Esposito, B., Cohen, J.L., Fisson, S., et al. (2006). Natural regulatory T cells control the development of atherosclerosis in mice. *Nature Medicine* 12, 178–180.
- Allan, S., Crome, S., and International ..., C.N. (2007). Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production.
- Anderson, M.S., and Su, M.A. (2016). AIRE expands: new roles in immune tolerance and beyond. *Nature Reviews. Immunology* 16, 247–258.
- Anderson, R., and Rapoport, B.L. (2018). Immune Dysregulation in Cancer Patients Undergoing Immune Checkpoint Inhibitor Treatment and Potential Predictive Strategies for Future Clinical Practice. *Frontiers in Oncology* 8, 80.
- Andersson, J., Libby, P., and Hansson, G.K. (2010). Adaptive immunity and atherosclerosis. *Clinical Immunology (Orlando, Fla.)* 134, 33–46.
- Arpaia, N., Green, J.A., Moltedo, B., Arvey, A., Hemmers, S., Yuan, S., Treuting, P.M., and Rudensky, A.Y. (2015). A Distinct Function of Regulatory T Cells in Tissue Protection. *Cell* 1078–1089.
- Arstila, T., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. (2000). Diversity of Human $\alpha\beta$ T Cell Receptors. *Science* 288, 1135–1135.
- Asseman, C., Mauze, S., Leach, M., Coffman, R., and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *The Journal of Experimental Medicine* 190, 995–1004.
- Baecher-Allan, C., Brown, J., Freeman, G., and Hafler, D. (2001). CD4+CD25^{high} regulatory cells in human peripheral blood. *Journal of Immunology (Baltimore, Md. : 1950)* 167, 1245–1253.
- Bayer, A.L., Lee, J.Y., de la Barrera, A., Surh, C.D., and Malek, T.R. (2008). A function for IL-7R for CD4+CD25+Foxp3+ T regulatory cells. *Journal of Immunology (Baltimore, Md. : 1950)* 181, 225–234.

- Bennett, C., Christie, J., and Ramsdell, F. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3.
- Bettelli, E., Dastrange, M., and Oukka, M. (2005). Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proceedings of the National Academy of Sciences of the United States of America* 102, 5138–5143.
- Bluestone, J.A. (1997). Is CTLA-4 a master switch for peripheral T cell tolerance? *The Journal of Immunology*.
- Booth, N.J., McQuaid, A.J., Sobande, T., Kissane, S., Agius, E., Jackson, S.E., Salmon, M., Falciani, F., Yong, K., Rustin, M.H., et al. (2010). Different proliferative potential and migratory characteristics of human CD4⁺ regulatory T cells that express either CD45RA or CD45RO. *Journal of Immunology (Baltimore, Md. : 1950)* 184, 4317–4326.
- Brunet, J.-F., Denizot, F., Luciani, M.-F., Roux-Dosseto, M., Suzan, M., Mattei, M.-G., and Golstein, P. (1987). A new member of the immunoglobulin superfamily—CTLA-4. *Nature* 328, 328267a0.
- Brunkow, M., Jeffery, E., Hjerrild, K., Paepers, B., Clark, L., Yasayko, S., Wilkinson, J., Galas, D., Ziegler, S., and Ramsdell, F. (2001). Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* 27, 68–73.
- Burchill, M.A., Yang, J., Vogtenhuber, C., Blazar, B.R., and Farrar, M.A. (2007). IL-2 Receptor β -Dependent STAT5 Activation Is Required for the Development of Foxp3⁺ Regulatory T Cells. *The Journal of Immunology* 178, 280–290.
- Burchill, M.A., Yang, J., Vang, K.B., Moon, J.J., Chu, H.H., Lio, C.-W.J., Vegoe, A.L., Hsieh, C.-S., Jenkins, M.K., and Farrar, M.A. (2008). Linked T Cell Receptor and Cytokine Signaling Govern the Development of the Regulatory T Cell Repertoire. *Immunity* 28, 112–121.
- Byun, D.J., Wolchok, J.D., Rosenberg, L.M., and Girotra, M. (2017). Cancer immunotherapy — immune checkpoint blockade and associated endocrinopathies. *Nature Reviews Endocrinology* 13, 195–207.
- Cederbom, L., Hall, H., and Ivars, F. (2000). CD4⁺CD25⁺ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *European Journal of Immunology* 30, 1538–1543.

Céfalı, D., Schneider, H., Matangkasombut, O., Kang, H., Brody, J., and Rudd, C. (1998). CD28 receptor endocytosis is targeted by mutations that disrupt phosphatidylinositol 3-kinase binding and costimulation. *J Immunol Baltim Md 1950* **160**, 2223–2230.

Chae, W.-J.J., Henegariu, O., Lee, S.-K.K., and Bothwell, A.L. (2006). The mutant leucine-zipper domain impairs both dimerization and suppressive function of Foxp3 in T cells. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 9631–9636.

Chambers, C., Sullivan, T., and Immunity, A.J. (1997). Lymphoproliferation in CTLA-4-deficient mice is mediated by costimulation-dependent activation of CD4+ T cells.

Chamoto, K., Al-Habsi, M., and Honjo, T. (2017). Role of PD-1 in Immunity and Diseases. *Current Topics in Microbiology and Immunology* **410**, 75–97.

Chatila, T., Blaeser, F., Ho, N., Lederman, H., Voulgaropoulos, C., Helms, C., and Bowcock, A. (2000). JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *The Journal of Clinical Investigation* **106**, R75-81.

Chikuma, S., Abbas, A.K., and Bluestone, J.A. (2005). B7-independent inhibition of T cells by CTLA-4. *Journal of Immunology (Baltimore, Md. : 1950)* **175**, 177–181.

Choi, J.-M.M., Ahn, M.-H.H., Chae, W.-J.J., Jung, Y.-G.G., Park, J.-C.C., ng, H.-M., Kim, Y.-E.E., Shin, J.-A.A., Park, C.-S.S., Park, J.-W.W., et al. (2006). Intranasal delivery of the cytoplasmic domain of CTLA-4 using a novel protein transduction domain prevents allergic inflammation. *Nature Medicine* **12**, 574–579.

de Coaña, Y., Choudhury, A., and Kiessling, R. (2015). Checkpoint blockade for cancer therapy: revitalizing a suppressed immune system. *Trends in Molecular Medicine* **21**, 482–491.

Cooper, M.D., and Alder, M.N. (2006). The Evolution of Adaptive Immune Systems. *Cell* **124**, 815–822.

Curran, M.A., Montalvo, W., Yagita, H., and Allison, J.P. (2010). PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 4275–4280.

Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.-F., Enjoji, K., Linden, J., Oukka, M., et al. (2007). Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Medicine* **204**, 1257–1265.

deLeeuw, R.J., Kost, S.E., Kakal, J.A., and Nelson, B.H. (2012). The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research* 18, 3022–3029.

Deng, G., Xiao, Y., Zhou, Z., Nagai, Y., Zhang, H., Li, B., and Greene, M.I. (2012). Molecular and biological role of the FOXP3 N-terminal domain in immune regulation by T regulatory/suppressor cells. *Experimental and Molecular Pathology* 93, 334–338.

Dieckmann, D., Plottner, H., Berchtold, S., Berger, T., and Schuler, G. (2001). Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *The Journal of Experimental Medicine* 193, 1303–1310.

Dominguez-Villar, M., and Hafler, D.A. (2018). Regulatory T cells in autoimmune disease. *Nature Immunology* 19, 665–673.

Doria, A., Zen, M., Bettio, S., Gatto, M., Bassi, N., Nalotto, L., Ghirardello, A., Iaccarino, L., and Punzi, L. (2012). Autoinflammation and autoimmunity: Bridging the divide. *Autoimmun Rev* 12, 22–30.

Du, J., Huang, C., Zhou, B., and Ziegler, S.F. (2008). Isoform-specific inhibition of ROR alpha-mediated transcriptional activation by human FOXP3. *Journal of Immunology (Baltimore, Md. : 1950)* 180, 4785–4792.

Duarte, J.H., Zelenay, S., Bergman, M.-L.L., Martins, A.C., and Demengeot, J. (2009). Natural Treg cells spontaneously differentiate into pathogenic helper cells in lymphopenic conditions. *European Journal of Immunology* 39, 948–955.

Dwyer, K., Deaglio, S., Gao, W., Friedman, D., Strom, T.B., and Robson, S.C. (2007). CD39 and control of cellular immune responses. *Purinergic Signalling* 3, 171–180.

Floess, S., Freyer, J., Siewert, C., Baron, U., Olek, S., Polansky, J., Schlawe, K., Chang, H.-D.D., Bopp, T., Schmitt, E., et al. (2007). Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biology* 5, e38.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology* 4, 330–336.

Fontenot, J.D., Rasmussen, J.P., Gavin, M.A., and Rudensky, A.Y. (2005a). A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nature Immunology* 6, 1142–1151.

Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y.

(2005b). Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22, 329–341.

Freeman, G., Gray, G., and Gimmi, C. (1991). Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7.

Gabrilovich, D.I., and Nagaraj, S. (2009). Myeloid-derived suppressor cells as regulators of the immune system. *Nature Reviews Immunology* 9, 162–174.

Gambineri, E., Mannurita, S., Hagin, D., Vignoli, M., Anover-Sombke, S., DeBoer, S., Segundo, G.R., Allenspach, E.J., Favre, C., Ochs, H.D., et al. (2018). Clinical, Immunological, and Molecular Heterogeneity of 173 Patients With the Phenotype of Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) Syndrome. *Frontiers in Immunology* 9, 2411.

Gershon, R., and Kondo, K. (1970). Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* 18, 723–737.

Godfrey, V., Wilkinson, J., Rinchik, E., and Russell, L. (1991a). Fatal lymphoreticular disease in the scurfy (sf) mouse requires T cells that mature in a sf thymic environment: potential model for thymic education. *Proceedings of the National Academy of Sciences of the United States of America* 88, 5528–5532.

Godfrey, V., Wilkinson, J., and Russell, L. (1991b). X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *The American Journal of Pathology* 138, 1379–1387.

Gottschalk, R.A., Corse, E., and Allison, J.P. (2012). Expression of Helios in peripherally induced Foxp3⁺ regulatory T cells. *Journal of Immunology (Baltimore, Md. : 1950)* 188, 976–980.

Grivnenkov, S.I., Greten, F.R., and Karin, M. (2010). *Immunity, Inflammation, and Cancer*. *Cell* 140.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of Cancer: The Next Generation. *Cell* 144.

Hara, M., Kingsley, C., Niimi, M., Read, S., Turvey, S., Bushell, A., Morris, P., Powrie, F., and Wood, K. (2001). IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *Journal of Immunology (Baltimore, Md. : 1950)* 166, 3789–3796.

Harper, K., Balzano, C., and Rouvier, E. (1991). CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and

Hill, J.A., Feuerer, M., Tash, K., Haxhinasto, S., Perez, J., Melamed, R., Mathis, D., and Benoist, C. (2007). Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 27, 786–800.

Himmel, M.E., MacDonald, K.G., Garcia, R.V., Steiner, T.S., and Levings, M.K. (2013). Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. *Journal of Immunology* (Baltimore, Md. : 1950) 190, 2001–2008.

Hoffmann, P., Eder, R., Kunz-Schughart, L.A., Andreesen, R., and Edinger, M. (2004). Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. *Blood* 104, 895–903.

Hori, S. (2012). The Foxp3 interactome: a network perspective of T(reg) cells. *Nature Immunology* 13, 943–945.

Hori, S., Nomura, T., and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* (New York, N.Y.) 299, 1057–1061.

Hou, T.Z., Qureshi, O.S., Wang, C.J., Baker, J., Young, S.P., Walker, L.S., and Sansom, D. (2015). A transendocytosis model of CTLA-4 function predicts its suppressive behavior on regulatory T cells. *Journal of Immunology* (Baltimore, Md. : 1950) 194, 2148–2159.

Hou, T.Z., Verma, N., Wanders, J., Kennedy, A., Soskic, B., Janman, D., Halliday, N., Rowshanravan, B., Worth, A., Qasim, W., et al. (2017). Identifying functional defects in patients with immune dysregulation due to LRBA and CTLA-4 mutations. *Blood* 129, 1458–1468.

Hsieh, C.-S.S., Liang, Y., Tyznik, A.J., Self, S.G., Liggitt, D., and Rudensky, A.Y. (2004). Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity* 21, 267–277.

Huehn, J., and Beyer, M. (2015). Epigenetic and transcriptional control of Foxp3+ regulatory T cells. *Seminars in Immunology* 27, 10–18.

Huehn, J., Polansky, J.K., and Hamann, A. (2009). Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nature Reviews. Immunology* 9, 83–89.

Huurman, V., Unger, W., Koeleman, B., Oaks, M., Chandraker, A., Terpstra, O., and Roep, B. (2007). Differential inhibition of autoreactive memory- and alloreactive naive T cell responses by soluble cytotoxic T lymphocyte antigen 4 (sCTLA4), CTLA4Ig and LEA29Y. *Clinical and Experimental Immunology* 150, 487–493.

Ichinose, K., Zhang, Z., Koga, T., Juang, Y.-T.T., Kis-Tóth, K., Sharpe, A.H., Kuchroo, V., Crispín, J.C., and Tsokos, G.C. (2013). Brief report: increased expression of a short splice variant of CTLA-4 exacerbates lupus in MRL/lpr mice. *Arthritis and Rheumatism* 65, 764–769.

Ichiyama, K., Yoshida, H., Wakabayashi, Y., Chinen, T., Saeki, K., Nakaya, M., Takaesu, G., Hori, S., Yoshimura, A., and Kobayashi, T. (2008). Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. *The Journal of Biological Chemistry* 283, 17003–17008.

Janeway, C. (1992). The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunology Today* 13, 11–16.

Joly, A.-L.L., Liu, S., Dahlberg, C.I., Mailer, R.K., Westerberg, L.S., and Andersson, J. (2015). Foxp3 lacking exons 2 and 7 is unable to confer suppressive ability to regulatory T cells in vivo. *Journal of Autoimmunity* 63, 23–30.

Jordan, Boesteanu, A., Reed, A., and Nature ..., P.A. (2001). Thymic selection of CD4+CD25+regulatory T cells induced by an agonist self-peptide.

Josefowicz, S.Z., Niec, R.E., Kim, H.Y., Treuting, P., Chinen, T., Zheng, Y., Umetsu, D.T., and Rudensky, A.Y. (2012). Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature* 482, 395–399.

Kanamori, M., Nakatsukasa, H., Okada, M., Lu, Q., and Yoshimura, A. (2016). Induced Regulatory T Cells: Their Development, Stability, and Applications. *Trends in Immunology* 37, 803–811.

Kaur, G., Goodall, J.C., Jarvis, L.B., and Gaston, H.J. (2010). Characterisation of Foxp3 splice variants in human CD4+ and CD8+ T cells--identification of Foxp3 Δ 7 in human regulatory T cells. *Molecular Immunology* 48, 321–332.

Keir, M.E., Butte, M.J., Freeman, G.J., and Sharpe, A.H. (2008). PD-1 and its ligands in tolerance and immunity. *Annual Review of Immunology* 26, 677–704.

Kim, H.-P.P., and Leonard, W.J. (2007). CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *The Journal of Experimental Medicine* 204, 1543–1551.

Kitagawa, Y., Wing, J.B., and Sakaguchi, S. (2015a). Transcriptional and Epigenetic Control of Regulatory T Cell Development. *Progress in Molecular Biology and Translational Science* 136, 1–33.

Kitagawa, Y., Ohkura, N., and Sakaguchi, S. (2015b). Epigenetic control of thymic Treg-cell

development. *European Journal of Immunology* 45, 11–16.

Klein, L., Kyewski, B., Allen, P.M., and Hogquist, K.A. (2014). Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). *Nature Reviews. Immunology* 14, 377–391.

Klocke, K., Sakaguchi, S., Holmdahl, R., and Wing, K. (2016). Induction of autoimmune disease by deletion of CTLA-4 in mice in adulthood. *Proceedings of the National Academy of Sciences of the United States of America* 113, E2383-92.

Kobie, J.J., Shah, P.R., Yang, L., Rebhahn, J.A., Fowell, D.J., and Mosmann, T.R. (2006). T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *Journal of Immunology (Baltimore, Md. : 1950)* 177, 6780–6786.

Koh, K.P., Sundrud, M.S., and Rao, A. (2009). Domain requirements and sequence specificity of DNA binding for the forkhead transcription factor FOXP3. *PloS One* 4, e8109.

Komatsu, N., Okamoto, K., Sawa, S., Nakashima, T., Oh-hora, M., Kodama, T., Tanaka, S., Bluestone, J.A., and Takayanagi, H. (2014). Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. *Nature Medicine* 20, 62–68.

Kong, K.-F., Fu, G., Zhang, Y., Yokosuka, T., Casas, J., Canonigo-Balancio, A.J., Becart, S., Kim, G., III, J.R., Kronenberg, M., et al. (2014). Protein kinase C- η controls CTLA-4-mediated regulatory T cell function. *Nature Immunology* 15, 465–472.

Kumar, B.V., Connors, T.J., and Farber, D.L. (2018). Human T Cell Development, Localization, and Function throughout Life. *Immunity* 48, 202–213.

Kündig, T.M., Shahinian, A., Kawai, K., Mittrücker, H.-W., Sebзда, E., Bachmann, M.F., Mak, T.W., and Ohashi, P.S. (1996). Duration of TCR Stimulation Determines Costimulatory Requirement of T Cells. *Immunity* 5, 41–52.

Kurd, N., and Robey, E.A. (2016). T-cell selection in the thymus: a spatial and temporal perspective. *Immunological Reviews* 271, 114–126.

Levings, M., Sangregorio, R., and Roncarolo, M. (2001). Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *The Journal of Experimental Medicine* 193, 1295–1302.

Li, Z., Li, D., Tsun, A., and Li, B. (2015). FOXP3+ regulatory T cells and their functional regulation. *Cellular & Molecular Immunology* 12, 558–565.

Liang, S.C., Greenwald, R.J., Latchman, Y.E., Rosas, L., Satoskar, A., Freeman, G.J., and Sharpe, A.H. (2006). PD-L1 and PD-L2 have distinct roles in regulating host immunity to cutaneous leishmaniasis. *European Journal of Immunology* 36, 58–64.

Liao, W., Schones, D., Oh, J., Cui, Y., Cui, K., and Nature ..., R.T. (2008). Priming for T helper type 2 differentiation by interleukin 2–mediated induction of interleukin 4 receptor α -chain expression.

Liao, W., Lin, J.-X.X., and Leonard, W.J. (2011). IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Current Opinion in Immunology* 23, 598–604.

Ling, V., Wu, P.W., Finnerty, H.F., Sharpe, A.H., Gray, G.S., and Collins, M. (1999). Complete Sequence Determination of the Mouse and Human CTLA4 Gene Loci: Cross-Species DNA Sequence Similarity beyond Exon Borders. *Genomics* 60, 341–355.

Linsley, P., Brady, W., and Urnes, M. (1991a). CTLA-4 is a second receptor for the B cell activation antigen B7.

Linsley, P., Brady, W., Grosmaire, L., Aruffo, A., Damle, N., and Ledbetter, J. (1991b). Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Medicine* 173, 721–730.

Lio, C.-W.W., and Hsieh, C.-S.S. (2008). A two-step process for thymic regulatory T cell development. *Immunity* 28, 100–111.

Liu, C., Workman, C.J., and Vignali, D.A. (2016). Targeting regulatory T cells in tumors. *The FEBS Journal* 283, 2731–2748.

Liu, S.M., Sutherland, A.P., Zhang, Z., Rainbow, D.B., Quintana, F.J., Paterson, A.M., Sharpe, A.H., Oukka, M., Wicker, L.S., and Kuchroo, V.K. (2012). Overexpression of the Ctla-4 isoform lacking exons 2 and 3 causes autoimmunity. *Journal of Immunology (Baltimore, Md. : 1950)* 188, 155–162.

Liu, W., Putnam, A.L., Xu-Yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., de Groth, B., et al. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *The Journal of Experimental Medicine* 203, 1701–1711.

Lopes, J., Torgerson, T., and of ..., S.L. (2006). Analysis of FOXP3 reveals multiple domains required for its function as a transcriptional repressor.

Lozano, T., Casares, N., and Lasarte, J.J. (2013). Searching for the Achilles Heel of FOXP3.

Frontiers in Oncology 3, 294.

Lu, L., Barbi, J., and Pan, F. (2017). The regulation of immune tolerance by FOXP3. *Nature Reviews. Immunology* 17, 703–717.

Lundberg, A.K., Jonasson, L., Hansson, G.K., and Mailer, R. (2017). Activation-induced FOXP3 isoform profile in peripheral CD4⁺ T cells is associated with coronary artery disease. *Atherosclerosis* 267, 27–33.

Lythe, G., Callard, R.E., Hoare, R.L., and Molina-París, C. (2016). How many TCR clonotypes does a body maintain? *J Theor Biol* 389, 214–224.

Mahmud, S.A., Manlove, L.S., hmitz, H., Xing, Y., Wang, Y., Owen, D.L., henkel, J., Boomer, J.S., Green, J.M., Yagita, H., et al. (2014). Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nature Immunology* 15, 473–481.

Mailer, R.K., Falk, K., and Rötzschke, O. (2009). Absence of leucine zipper in the natural FOXP3Delta2Delta7 isoform does not affect dimerization but abrogates suppressive capacity. *PLoS One* 4, e6104.

Mailer, R.K., Joly, A.-L.L., Liu, S., Elias, S., Tegner, J., and Andersson, J. (2015). IL-1 β promotes Th17 differentiation by inducing alternative splicing of FOXP3. *Scientific Reports* 5, 14674.

Mantel, P.-Y.Y., Ouaked, N., Rückert, B., Karagiannidis, C., Welz, R., Blaser, K., and Schmidt-Weber, C.B. (2006). Molecular mechanisms underlying FOXP3 induction in human T cells. *Journal of Immunology (Baltimore, Md. : 1950)* 176, 3593–3602.

Marson, A., Kretschmer, K., Frampton, G.M., Jacobsen, E.S., Polansky, J.K., MacIsaac, K.D., Levine, S.S., Fraenkel, E., von Boehmer, H., and Young, R.A. (2007). Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* 445, 931–935.

Mavin, E., Nicholson, L., Ahmed, S., Gao, F., Dickinson, A., and Wang, X.-N.N. (2017). Human Regulatory T Cells Mediate Transcriptional Modulation of Dendritic Cell Function. *Journal of Immunology (Baltimore, Md. : 1950)* 198, 138–146.

van der Merwe, P., Bodian, D., Daenke, S., Linsley, P., and Davis, S. (1997). CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *The Journal of Experimental Medicine* 185, 393–403.

Misra, N., Bayry, J., Lacroix-Desmazes, S., Kazatchkine, M.D., and ni Kaveri, V. (2004). Cutting edge: human CD4⁺CD25⁺ T cells restrain the maturation and antigen-presenting function of dendritic cells. *Journal of Immunology (Baltimore, Md. : 1950)* 172, 4676–4680.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., et al. (2009). Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity* 30, 899–911.

Miyara, M., Chader, D., Sage, E., Sugiyama, D., Nishikawa, H., Bouvry, D., Claër, L., Hingorani, R., Balderas, R., Rohrer, J., et al. (2015). Sialyl Lewis x (CD15s) identifies highly differentiated and most suppressive FOXP3^{high} regulatory T cells in humans. *Proceedings of the National Academy of Sciences of the United States of America* 112, 7225–7230.

Möller, G. (1988). Do Suppressor T Cells Exist? *Scand J Immunol* 27, 247–250.

Monaco, G., Lee, B., Xu, W., Mustafah, S., Hwang, Y.Y., Carré, C., Burdin, N., Visan, L., Ceccarelli, M., Poidinger, M., et al. (2019). RNA-Seq Signatures Normalized by mRNA Abundance Allow Absolute Deconvolution of Human Immune Cell Types. *Cell Reports* 26, 1627-1640.e7.

mstein, R., Josefowicz, S.Z., Arvey, A., Treuting, P.M., and Rudensky, A.Y. (2012a). Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell* 150, 29–38.

mstein, R., Arvey, A., Josefowicz, S.Z., Peng, X., Reynolds, A., Sandstrom, R., Neph, S., Sabo, P., Kim, J.M., Liao, W., et al. (2012b). Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification. *Cell* 151, 153–166.

Murphy, K., and Weaver, C. (2017). Janeway's immunobiology, 9th edition.

Naluai, Å., Nilsson, S., Samuelsson, L., Gudjónsdóttir, A.H., Ascher, H., Ek, J., Hallberg, B., Kristiansson, B., Martinsson, T., Nerman, O., et al. (2000). The CTLA4/CD28 gene region on chromosome 2q33 confers susceptibility to celiac disease in a way possibly distinct from that of type 1 diabetes and other chronic inflammatory disorders. *Tissue Antigens* 56, 350–355.

Natoli, G., and Ostuni, R. (2019). Adaptation and memory in immune responses. *Nature Immunology* 20, 783–792.

Nie, J., Li, Y.Y., Zheng, S.G., Tsun, A., and Li, B. (2015). FOXP3(+) Treg Cells and Gender Bias in Autoimmune Diseases. *Frontiers in Immunology* 6, 493.

van Nieuwenhuijze, A., and Liston, A. (2015). The Molecular Control of Regulatory T Cell Induction. *Progress in Molecular Biology and Translational Science* 136, 69–97.

Nishikawa, Y., Nishijima, H., Matsumoto, M., Morimoto, J., Hirota, F., Takahashi, S., Luche,

H., Fehling, H., Mouri, Y., and Matsumoto, M. (2014). Temporal Lineage Tracing of Aire-Expressing Cells Reveals a Requirement for Aire in Their Maturation Program. *J Immunol* 192, 2585–2592.

Nishizuka, Y., and Sakakura, T. (1969). Thymus and reproduction: sex-linked dysgenesis of the gonad after neonatal thymectomy in mice. *Science (New York, N.Y.)* 166, 753–755.

Oaks, M., Hallett, K., Penwell, R., Stauber, E., Warren, S., and Tector, A. (2000). A native soluble form of CTLA-4. *Cellular Immunology* 201, 144–153.

Ogawa, C., Tone, Y., Tsuda, M., Peter, C., Waldmann, H., and Tone, M. (2014). TGF- β -mediated Foxp3 gene expression is cooperatively regulated by Stat5, Creb, and AP-1 through CNS2. *Journal of Immunology (Baltimore, Md. : 1950)* 192, 475–483.

Ohue, Y., and Nishikawa, H. (2019). Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Science* 110, 2080–2089.

Okazaki, T., Okazaki, I., Wang, J., Sugiura, D., Nakaki, F., Yoshida, T., Kato, Y., Fagarasan, S., Muramatsu, M., Eto, T., et al. (2011). PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. *The Journal of Experimental Medicine* 208, 395–407.

Onishi, Y., Fehervari, Z., Yamaguchi, T., and Sakaguchi, S. (2008). Foxp3⁺ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proceedings of the National Academy of Sciences of the United States of America* 105, 10113–10118.

Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T., and Nakayama, E. (1999). Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. *Cancer Res* 59, 3128–3133.

Ono, M., Yaguchi, H., Ohkura, N., Kitabayashi, I., Nagamura, Y., Nomura, T., Miyachi, Y., Tsukada, T., and Sakaguchi, S. (2007). Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* 446, 685–689.

O'sullivan, T., Sun, J., and Immunity, L.L. (2015). Natural killer cell memory.

Owen, D.L., Mahmud, S.A., Sjaastad, L.E., Williams, J.B., Spanier, J.A., Simeonov, D.R., Ruscher, R., Huang, W., Proekt, I., Miller, C.N., et al. (2019). Thymic regulatory T cells arise via two distinct developmental programs. *Nature Immunology* 20, 195–205.

Pacholczyk, R., and Kern, J. (2008). The T-cell receptor repertoire of regulatory T cells. *Immunology* 125, 450–458.

Pacholczyk, R., Ignatowicz, H., Kraj, P., and Ignatowicz, L. (2006). Origin and T cell receptor diversity of Foxp3+CD4+CD25+ T cells. *Immunity* 25, 249–259.

Pandiyar, P., Zheng, L., Ishihara, S., Reed, J., and Lenardo, M.J. (2007). CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nature Immunology* 8, 1353–1362.

Passos, G.A., Speck-Hernandez, C.A., Assis, A.F., and Mendes-da-Cruz, D.A. (2018). Update on Aire and thymic negative selection. *Immunology* 153, 10–20.

Paterson, A.M., Lovitch, S.B., Sage, P.T., Juneja, V.R., Lee, Y., Trombly, J.D., Arancibia-Cárcamo, C.V., Sobel, R.A., Rudensky, A.Y., Kuchroo, V.K., et al. (2015). Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity. *The Journal of Experimental Medicine* 212, 1603–1621.

Perrot, I., Michaud, H.-A.A., Giraudon-Paoli, M., Augier, S., Docquier, A., Gros, L., Courtois, R., Déjou, C., Jecko, D., Becquart, O., et al. (2019). Blocking Antibodies Targeting the CD39/CD73 Immunosuppressive Pathway Unleash Immune Responses in Combination Cancer Therapies. *Cell Reports* 27, 2411-2425.e9.

Pesce, G., Auricchio, R., Bagnasco, M., and Saverino, D. (2014). Oversecretion of soluble CTLA-4 in various autoimmune diseases overlapping celiac disease. *Genetic Testing and Molecular Biomarkers* 18, 8–11.

Pieper, K., Grimbacher, B., and Eibel, H. (2013). B-cell biology and development. *Journal of Allergy and Clinical Immunology* 131, 959–971.

Polansky, J.K., Kretschmer, K., Freyer, J., Floess, S., Garbe, A., Baron, U., Olek, S., Hamann, A., von Boehmer, H., and Huehn, J. (2008). DNA methylation controls Foxp3 gene expression. *European Journal of Immunology* 38, 1654–1663.

Quandt, D., Hoff, H., Rudolph, M., Fillatreau, S., and Brunner-Weinzierl, M.C. (2007). A New Role of CTLA-4 on B Cells in Thymus-Dependent Immune Responses In Vivo. *J Immunol* 179, 7316–7324.

Qureshi, O.S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., hmidt, E., Baker, J., Jeffery, L.E., Kaur, S., Briggs, Z., et al. (2011). Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science (New York, N.Y.)* 332, 600–603.

Reichert, S.L., McKay, E.M., and Moldenhauer, J.S. (2016). Identification of a novel nonsense mutation in the FOXP3 gene in a fetus with hydrops--Expanding the phenotype of IPEX syndrome. *American Journal of Medical Genetics. Part A* 170A, 226–232.

Richards, D.M., Delacher, M., Goldfarb, Y., Kägebein, D., Hofer, A.-C.C., Abramson, J., and Feuerer, M. (2015). Treg Cell Differentiation: From Thymus to Peripheral Tissue. *Progress in Molecular Biology and Translational Science* 136, 175–205.

Rosenblum, M.D., Way, S.S., and Abbas, A.K. (2016). Regulatory T cell memory. *Nature Reviews. Immunology* 16, 90–101.

Round, J.L., and Mazmanian, S.K. (2010). Inducible Foxp3⁺ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proc National Acad Sci* 107, 12204–12209.

Ruan, Q., Kameswaran, V., Tone, Y., Li, L., Liou, H.-C.C., Greene, M.I., Tone, M., and Chen, Y.H. (2009). Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. *Immunity* 31, 932–940.

Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., et al. (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28, 546–558.

Rubtsov, Y.P., Niec, R.E., Josefowicz, S., Li, L., Darce, J., Mathis, D., Benoist, C., and Rudensky, A.Y. (2010). Stability of the regulatory T cell lineage in vivo. *Science (New York, N.Y.)* 329, 1667–1671.

Rudd, C.E., Taylor, A., and Schneider, H. (2009). CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunological Reviews* 229, 12–26.

Rudra, D., deRoos, P., Chaudhry, A., Niec, R.E., Arvey, A., mstein, R., Leslie, C., Shaffer, S.A., Goodlett, D.R., and Rudensky, A.Y. (2012). Transcription factor Foxp3 and its protein partners form a complex regulatory network. *Nature Immunology* 13, 1010–1019.

Sadlon, T.J., Wilkinson, B.G., Pederson, S., Brown, C.Y., Bresatz, S., Gargett, T., Melville, E.L., Peng, K., D’Andrea, R.J., Glonek, G.G., et al. (2010). Genome-wide identification of human FOXP3 target genes in natural regulatory T cells. *Journal of Immunology (Baltimore, Md. : 1950)* 185, 1071–1081.

Saeed, S., Quintin, J., Kerstens, H.H., Rao, N.A., Aghajaniirefah, A., Matarese, F., Cheng, S.-C., Ratter, J., Berentsen, K., and Ent, M.A. (2014). Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity. *Science* 345, 1251086.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.

Journal of Immunology (Baltimore, Md. : 1950) 155, 1151–1164.

Sakaguchi, S., Miyara, M., Costantino, C.M., and Hafler, D.A. (2010). FOXP3⁺ regulatory T cells in the human immune system. *Nature Reviews. Immunology* 10, 490–500.

Salomon, B., Lenschow, D., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4⁺CD25⁺ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12, 431–440.

Sansom, D., Manzotti, C.N., and Zheng, Y. (2003). What's the difference between CD80 and CD86? *Trends in Immunology* 24, 314–319.

Saverino, D., Simone, R., Bagnasco, M., and Pesce, G. (2010). The soluble CTLA-4 receptor and its role in autoimmune diseases: an update. *Auto- Immunity Highlights* 1, 73–81.

Schlenner, S.M., Weigmann, B., Ruan, Q., Chen, Y., and von Boehmer, H. (2012). Smad3 binding to the foxp3 enhancer is dispensable for the development of regulatory T cells with the exception of the gut. *J Exp Medicine* 209, 1529–1535.

Shahinian, A., Pfeffer, K., Lee, K., Kundig, T., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P., Thompson, C., and Mak, T. (1993). Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261, 609–612.

Simone, R., Pesce, G., Antola, P., Rumbullaku, M., Bagnasco, M., Bizzaro, N., and Saverino, D. (2014). The soluble form of CTLA-4 from serum of patients with autoimmune diseases regulates T-cell responses. *BioMed Research International* 2014, 215763.

Smith, E.L., Finney, H.M., Nesbitt, A.M., Ramsdell, F., and Robinson, M.K. (2006). Splice variants of human FOXP3 are functional inhibitors of human CD4⁺ T-cell activation. *Immunology* 119, 203–211.

Stumpf, M., Zhou, X., and Bluestone, J.A. (2013). The B7-independent isoform of CTLA-4 functions to regulate autoimmune diabetes. *Journal of Immunology (Baltimore, Md. : 1950)* 190, 961–969.

Stumpf, M., Zhou, X., Chikuma, S., and Bluestone, J.A. (2014). Tyrosine 201 of the cytoplasmic tail of CTLA-4 critically affects T regulatory cell suppressive function. *European Journal of Immunology* 44, 1737–1746.

Szurek, E., Cebula, A., Wojciech, L., Pietrzak, M., Rempala, G., Kisielow, P., and Ignatowicz, L. (2015). Differences in Expression Level of Helios and Neuropilin-1 Do Not Distinguish Thymus-Derived from Extrathymically-Induced CD4⁺Foxp3⁺ Regulatory T Cells. *PloS One* 10, e0141161.

- Taams, L.S., Stejic, M., Smith, J., Dunne, P.J., Fletcher, J.M., Plunkett, F.J., Ebeling, S.B., Lombardi, G., Rustin, M.H., and Bijlsma, J.W. (2002). Antigen specific T cell suppression by human CD4⁺ CD25⁺ regulatory T cells. *European Journal of Immunology* 32, 1621–1630.
- Tai, X., Cowan, M., Feigenbaum, L., and Singer, A. (2005). CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nature Immunology* 6, 152–162.
- Tai, X., Laethem, F., Sharpe, A.H., and Singer, A. (2007). Induction of autoimmune disease in CTLA-4^{-/-} mice depends on a specific CD28 motif that is required for in vivo costimulation. *Proceedings of the National Academy of Sciences of the United States of America* 104, 13756–13761.
- Takaba, H., and Takayanagi, H. (2017). The Mechanisms of T Cell Selection in the Thymus. *Trends in Immunology* 38, 805–816.
- Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T.W., and Sakaguchi, S. (2000). Immunologic Self-Tolerance Maintained by Cd25⁺Cd4⁺Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte–Associated Antigen 4. *The Journal of Experimental Medicine* 192, 303–310.
- Tang, Q., Adams, J.Y., Tooley, A.J., Bi, M., Fife, B.T., Serra, P., Santamaria, P., Locksley, R.M., Krummel, M.F., and Bluestone, J.A. (2006). Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nature Immunology* 7, 83–92.
- Teng, M., Ngiow, S., von Scheidt, B., McLaughlin, N., Sparwasser, T., and Smyth, M.J. (2010). Conditional Regulatory T-Cell Depletion Releases Adaptive Immunity Preventing Carcinogenesis and Suppressing Established Tumor Growth. *Cancer Res* 70, 7800–7809.
- Thornton, A., and Shevach, E. (1998). CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *The Journal of Experimental Medicine* 188, 287–296.
- Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., and Shevach, E. (2010). Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3⁺ T regulatory cells. *Journal of Immunology* (Baltimore, Md. : 1950) 184, 3433–3441.
- Tivol, E., Borriello, F., Schweitzer, A., Lynch, W., Bluestone, J., and Sharpe, A. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3, 541–547.

Tran, D., Ramsey, H., and Eevach (2007). Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor- dependent but does not confer a regulatory phenotype. *Blood* 110, 2983–2990.

Tseng, S., Otsuji, M., Gorski, K., and of ..., H.X. (2001). B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells.

Ueda, H., Howson, J.M., Esposito, L., Heward, J., Snook, H., Chamberlain, G., Rainbow, D.B., Hunter, K.M., Smith, A.N., Genova, G., et al. (2003). Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423, 506–511.

Vang, K., Yang, J., Pagán, A., and of ..., L.L. (2010). Cutting edge: CD28 and c-Rel–dependent pathways initiate regulatory T cell development.

Vignali, D.A., Collison, L.W., and Workman, C.J. (2008). How regulatory T cells work. *Nature Reviews. Immunology* 8, 523–532.

Vijayakrishnan, L., avik, J., Illés, Z., Greenwald, R.J., Rainbow, D., Greve, B., Peterson, L.B., Hafler, D.A., Freeman, G.J., Sharpe, A.H., et al. (2004). An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity* 20, 563–575.

Vogel, C., de Abreu, R., Ko, D., Le, S., Shapiro, B.A., Burns, S.C., Sandhu, D., Boutz, D.R., Marcotte, E.M., and Penalva, L.O. (2010). Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. *Mol Syst Biol* 6, 400.

Walker, L.S., and Sansom, D. (2011). The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nature Reviews. Immunology* 11, 852–863.

Walker, M.R., Kasproicz, D.J., Gersuk, V.H., Benard, A., Landeghen, M., Buckner, J.H., and Ziegler, S.F. (2003). Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *The Journal of Clinical Investigation* 112, 1437–1443.

Walunas, T., Lenschow, D., Bakker, C., Linsley, P., Freeman, G., Green, J., Thompson, C., and Bluestone, J. (1994). CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1, 405–413.

Wang, X.B., Fan, Z.Z., Anton, D., Vollenhoven, A.V., Ni, Z.H., Chen, X.F., and Lefvert, A.K. (2011). CTLA4 is expressed on mature dendritic cells derived from human monocytes and influences their maturation and antigen presentation. *Bmc Immunol* 12, 21.

Waterhouse, P., Penninger, J., Timms, E., Wakeham, A., Shahinian, A., Lee, K., Thompson, C., Griesser, H., and Mak, T. (1995). Lymphoproliferative disorders with early lethality in mice

deficient in Ctla-4. *Science (New York, N.Y.)* 270, 985–988.

Weiss, J.M., Bilate, A.M., Gobert, M., Ding, Y., de Lafaille, M.A., Parkhurst, C.N., Xiong, H., Dolpady, J., Frey, A.B., Ruocco, M.G., et al. (2012). Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *The Journal of Experimental Medicine* 209, 1723–1742, S1.

Whiteside, T. (2008). The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 27, onc2008271.

Wildin, R., Ramsdell, F., Peake, J., and Faravelli, F. (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy.

Winerdal, M.E., Krantz, D., Hartana, C.A., Zirakzadeh, A.A., Linton, L., Bergman, E.A., Rosenblatt, R., Vasko, J., Alamdari, F., Hansson, J., et al. (2018). Urinary Bladder Cancer Tregs Suppress MMP2 and Potentially Regulate Invasiveness. *Cancer Immunol Res* 6, canimm.0466.2017.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. *Science (New York, N.Y.)* 322, 271–275.

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A.D., Stroud, J.C., Bates, D.L., Guo, L., Han, A., Ziegler, S.F., et al. (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 126, 375–387.

Xavier-da-Silva, M.M., Moreira-Filho, C.A., Suzuki, E., Patricio, F., Coutinho, A., and Carneiro-Sampaio, M. (2015). Fetal-onset IPEX: report of two families and review of literature. *Clinical Immunology (Orlando, Fla.)* 156, 131–140.

Yadav, M., Louvet, C., Davini, D., Gardner, J.M., Martinez-Llordella, M., Bailey-Bucktrout, S., Anthony, B.A., erdrup, F., Head, R., Kuster, D.J., et al. (2012). Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *The Journal of Experimental Medicine* 209, 1713–1722, S1-19.

Yamazaki, T., Akiba, H., Iwai, H., and of ..., M.H. (2002a). Expression of programmed death 1 ligands by murine T cells and APC.

Yamazaki, T., Akiba, H., Iwai, H., Matsuda, H., Aoki, M., Tanno, Y., Shin, T., Tsuchiya, H., Pardoll, D.M., Okumura, K., et al. (2002b). Expression of programmed death 1 ligands by murine T cells and APC. *Journal of Immunology (Baltimore, Md. : 1950)* 169, 5538–5545.

Yang, X.O., Nurieva, R., Martinez, G.J., Kang, H., Chung, Y., Pappu, B.P., Shah, B., Chang, S., Schluns, K.S., Watowich, S.S., et al. (2008). Molecular Antagonism and Plasticity of Regulatory and Inflammatory T Cell Programs. *Immunity* 29, 44–56.

Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E., Moriggl, R., et al. (2007). Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109, 4368–4375.

Zarek, P.E., Huang, C.-T.T., Lutz, E.R., Kowalski, J., Horton, M.R., Linden, J., Drake, C.G., and Powell, J.D. (2008). A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* 111, 251–259.

Zhang, R., Huynh, A., Whitcher, G., Chang, J., Maltzman, J.S., and Turka, L.A. (2013). An obligate cell-intrinsic function for CD28 in Tregs. *The Journal of Clinical Investigation* 123, 580–593.

Zheng, Y., Josefowicz, S.Z., Kas, A., Chu, T.-T.T., Gavin, M.A., and Rudensky, A.Y. (2007). Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 445, 936–940.

Zheng, Y., Josefowicz, S., Chaudhry, A., Peng, X.P., Forbush, K., and Rudensky, A.Y. (2010). Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* 463, 808–812.

Zhou, L., Lopes, J.E., Chong, M.M., Ivanov, I.I., Min, R., Victora, G.D., Shen, Y., Du, J., Rubtsov, Y.P., Rudensky, A.Y., et al. (2008). TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgamma function. *Nature* 453, 236–240.